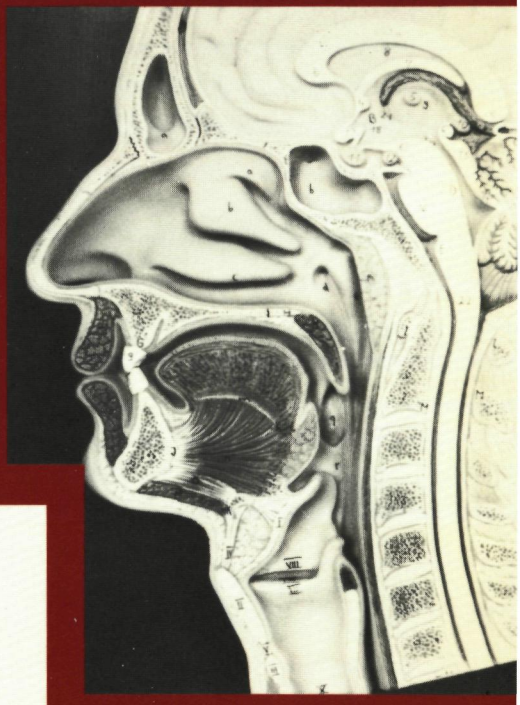
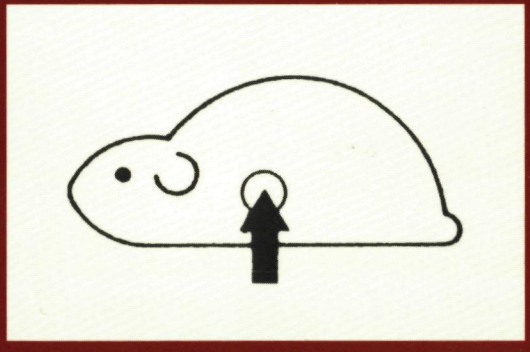
Locoregional immunotherapy with interleukin-2 in head and neck squamous-cell carcinoma

Preclinical and clinical studies





Vera Mattijssen

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Aan mijn ouders

Contents

Chapter 1

General introduction

Cancer is a major health problem in Western countries. About 50% of patients in whom cancer is diagnosed will die of this disease. As the second cause of death, it accounts for 22% of all deaths in the United States [1] and for 27% in The Netherlands [2].
Surgery, radiotherapy and chemotherapy are the keystones of cancer treatment. Recently, as a result of increased knowledge on host defense mechanisms and advanced biotechnologic developments, immunotherapy emerged as a fourth treatment modality of cancer [3]. Immunotherapy aims at stimulation of the host immune system to detect and to eliminate cancer cells. The immune system encompasses a range of immune-competent cells, such as T lymphocytes, B lymphocytes, natural killer (NK) cells, monocytes, macrophages and granulocytes, whose natural functions are regulated by soluble protein products of mainly lymphocytes and monocytes, called cytokines.

Interleukin-2

One of the first cytokines recognized was interleukin-2 [4]. This IL-2 has a pivotal role in the generation and regulation of the immune response [5]. IL-2 is secreted by T lymphocytes upon antigenic stimulation of the T cell receptor. Subsequently, it induces clonal expansion of antigen-selected T cells and B cells, and promotes the secretion of additional cytokines and antibodies. This is the basis for a specific immune response and immune memory. In addition, IL-2 may augment antigen-nonspecific immune reactivity by activation of NK cells and monocytes.
Because of its effects on a range of immune cells, IL-2 was supposed to be a valuable stimulant in anticancer immunotherapy. Immune cells might cause cancer-cell destruction in several ways, such as direct cell-mediated lysis, antibody-mediated lysis, and release of toxic mediators. At least two potential anticancer effector cells were identified *in vitro* using IL-2. By incubation of peripheral blood- or spleen lymphocytes with IL-2, lymphokine-activated killer (LAK) cells were generated, which were capable of lysing a broad range of fresh malignant, but not normal cells *in vitro*. On the other hand, expansion of tumor-infiltrating lymphocytes (TIL), isolated from solid tumors, with IL-2 *in vitro* resulted in cells with specific immune reactivity against the autologous tumor [3].
Experimental results concerning the anticancer immune-stimulating effects of IL-2 have been mainly translated into the development of clinical trials using high-dose intravenous IL-2 administration [6]. Objective responses were described in 15-30% of selected patients with metastasized melanoma or advanced renal cell carcinoma. The underlying mechanism of these IL-2-induced antitumor effects *in vivo* is not yet clear.
It is questionable whether systemic administration of high doses IL-2 is the most beneficial way to induce an antitumor immune response. Endogenous IL-2 acts over short distances,

at the site of antigen presentation, as a paracrine or autocrine mediator. Exogenous IL-2 is rapidly cleared from the circulation through the kidneys. Lymphocytes at the tumor site, which presumably have been sensitized against autologous tumor antigens, therefore probably will not be affected by intravenously administered IL-2. Furthermore, systemic high-dose IL-2 therapy is accompanied by serious toxicity.

MHC molecules

Major Histocompatibility (MHC) molecules are cell-surface glycoproteins which play an important role in antigen presentation towards T lymphocytes. In man, these MHC molecules are represented by human leukocyte antigens (HLA). Antigen-specific $CD8^+$ T lymphocytes recognize cell-surface antigens if they are presented together with MHC class I molecules, and $CD4^+$ T lymphocytes recognize antigens in combination with MHC class II molecules. In other words: immunorecognition by T lymphocytes is MHC-restricted. This means that direct interaction of specific T lymphocytes with tumor cells will depend upon the expression of MHC molecules by the tumor. It has been demonstrated that cancer cells may have altered MHC expression as compared to the originating normal tissue [7]. Both defective MHC class I expression and *de novo* MHC class II expression were found. Therefore, studying MHC expression by tumor cells *in vivo* is relevant in immunotherapeutical trials in which T lymphocytes are supposed to play a role. NK cells, LAK cells, and monocytes, on the other hand, interact with tumor cells in a MHC-nonrestricted way.

Head and neck squamous-cell carcinoma

Head and neck squamous-cell carcinomas (HNSCC) are derived from the upper aerodigestive tract, predominantly the oral cavity, larynx and pharynx. Most tumors occur in men over 50 years of age. They encompass about 5% of all malignant tumors in men, and about 2% in women [1]. HNSCC primarily metastasizes to the regional neck lymph nodes. Approximately one third of patients present with confined T1 and T2 lesions. Of these patients, more than 80% and 60%, respectively, will be cured by surgery or radiotherapy. Two thirds of the patients have locally or regionally advanced disease at presentation (T3 or T4, N1-3, M0). About 30% of them will be cured after extensive surgery and radiation therapy. The majority, however, will die of locally or regionally persistent, or recurrent disease [8]. Although induction chemotherapy in HNSCC results in very high response rates, a survival benefit of adding chemotherapy to standard surgery and radiotherapy has not been confirmed in randomized trials [9]. In recurrent HNSCC, chemotherapy has a palliative role only. This indicates that new treatment approaches are

desirable in this disease. In patients with HNSCC impaired systemic and regional immuno-reactivity is well documented [10]. Whether this dysfunction is pre-existing and causative, or tumor-induced is not clear. Depressed immunity in HNSCC patients has been correlated with poor prognosis [10]. Immunotherapy with IL-2 may be an alternative approach. Because of the locoregional tumor spread, accessibility to injection, and localization close to extensive submucosal and nodal lymphatic tissue, HNSCC appears especially suitable to locoregional IL-2 administration.

Outline of this study

This study was directed at exploration of the optimal route, schedule, and formulation of IL-2 in locoregional immunotherapy, and its local and systemic antitumor effects, in order to be applied in HNSCC. Besides, the mechanism of the induced antitumor effect was studied by evaluation of MHC expression on tumor cells and composition of the mononuclear cell infiltrate at the tumor site with immunohistochemical methods.

Chapter 2 outlines the biological and immune-stimulating effects of IL-2, and the rationale for low-dose locoregional administration in anticancer immunotherapy. Furthermore, an overview of the literature on locoregional IL-2 administration in experimental and clinical studies is provided.

Chapter 3 describes a study on the distribution of MHC class I and II expression in HNSCC using immunohistochemical methods. The staining procedures and scoring system established here, were applied in the evaluation of tumor samples obtained from HNSCC lesions treated with IL-2 in subsequent studies.

Our first clinical study (chapter 4) concerned low-dose perilymphatic IL-2 administration in patients with nonpretreated locally far-advanced HNSCC. The treatment protocol was based on the previously published preliminary results with local IL-2 injections in HNSCC [11]. Aims of this study were confirmation of these results, and evaluation of the local effects at the microscopical level.

Subsequently, an experimental study (chapter 5) was performed to further explore the optimal route, schedule, and formulation of IL-2 in locoregional immunotherapy of established tumors. For this goal, IL-2 and polyethylene glycol-modified IL-2 (PEG-IL-2) were applied in the guinea-pig line-10 carcinoma model, characterized by regional lymph-node metastases, and thus bearing resemblance to human HNSCC.

The results of serial histological and immunohistochemical analysis of line-10 tumor regression induced by intratumoral PEG-IL-2 injections are described in chapter 6.

Based on the experimental results in the line-10 tumor, another clinical protocol was developed to study the feasibility and efficacy of intratumoral PEG-IL-2 injections in patients with locoregionally recurrent HNSCC. The clinical and histopathological results obtained are represented in chapter 7.

We assumed that intratumoral injection of PEG-IL-2 might result in longer retention in the tumor and the tumor-draining lymph nodes than IL-2. To verify this, the fate of radiolabeled IL-2 and PEG-IL-2 following intratumoral injection was studied in the guinea-pig line-10 tumor (chapter 8).

In chapter 9 the results are summarized and discussed.

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Chapter 2

Immunotherapy of cancer with interleukin-2 with emphasis on locoregional supply: rationale, and overview of the literature

Introduction

An important question in tumor immunology is why overt spontaneous tumors are incapable of eliciting an immune response resulting in rejection of the tumor. Prerequisites for such a response would be the presence of specific tumor antigens, adequate presentation and recognition of these antigens, and the generation of an effector reaction. This implies that the non- or low immunogenicity of tumors can be both tumor- and host-related.

The insight into potential tumor antigens has substantially increased since it was found that cytotoxic T lymphocytes (CTL) can recognize short intracellular peptides, which are processed and presented at the cell surface in combination with major histocompatibility MHC) class I molecules [1]. Endogenously synthesized peptide products induced by genetic alterations in tumor cells thus can provide new epitopes to generate specific CTL [2], as was demonstrated for point mutations in murine tumor cells [3]. In humans, the infiltration of lymphocytes observed in many solid tumors is suggestive of a host-tumor interaction. These tumor-infiltrating lymphocytes (TIL) provide a reagent for detecting cellular immune responses to tumor antigens. In selected tumors, immune recognition was confirmed *in vitro* by specific MHC class I-restricted cytolytic activity of TIL against the autologous tumor [4], and by specific release of cytokines by TIL after incubation with the autologous tumor [5]. In addition, expression of memory T cell markers by TIL [6], as well as specific localization in tumor deposits after re-infusion [7] are suggestive of recognition of tumor antigens. Evidence for specific tumor antigens was obtained recently with the identification of the MAGE gene family in human melanomas [8]. These genes are expressed by different tumors and not by most normal cells. The antigen encoded for by MAGE-1 is recognized by anti-melanoma human leukocyte antigen (HLA)-A1-restricted CTL.

An effective tumor-host interaction also depends on a well-functioning host immune system. Impaired immune responsiveness has been demonstrated in tumor-bearing animals and cancer patients [9]. The origin and nature of the defects and the consequences for interaction with the tumor are incompletely understood. In mice progressive tumor growth is accompanied with further decreased cytotoxic activity of nonspecific immune cells and CTL [10,11].

Stimulation of the failing host immune system to detect and to eliminate tumor cells might provide a means of anticancer therapy. The T cell product interleukin-2 (IL-2) has a variety of immune-stimulating effects. Natural IL-2 plays an essential role in the initiation and amplification of an immune response. In experimental situations IL-2

promotes the proliferation and the antitumor cytolytic activity of specific and nonspecific immune cells. In this paper it will be discussed how these effects of IL-2 have been applied in the treatment of experimental and human cancer. With the biological function of IL-2 as a basis, especially locoregional administration will be outlined.

Biological activity of interleukin-2

IL-2 is a 15-Kd protein produced by T lymphocytes. As the first of a series of lymphocytotrophic hormones to be recognized [12] and characterized [13], it has had great impact on understanding the regulation of the immune response, in which it plays a pivotal role [14]. Resting T lymphocytes do not produce IL-2, nor do they respond to exogenously added IL-2. However, upon antigenic activation of the T cell receptor, T lymphocytes start producing IL-2, and express IL-2 receptors on their cell-surface. IL-2 promotes the clonal expansion, and enhances the secretory capacities of antigen-selected T cells [14] and B cells [15]. This is the basis for a specific immune response and immune memory. In addition, IL-2 augments antigen-nonspecific immune reactivity by activation of natural killer (NK) cells. Defective IL-2 production causes severe combined immunodeficiency disease [16,17].

Antigen presentation thus results in IL-2-mediated clonal proliferation of T cells and production of secondary cytokines. These cytokines are involved in the subsequent differentiation of immune effector cells. There is increasing evidence that functional T cell subsets can be defined by the pattern of cytokines they produce [18,19]. $CD4^+$ helper T cells, which recognize antigens presented in conjunction with MHC class II molecules, have been divided into T_H1 cells and T_H2 cells. T_H1 cells, secreting IL-2 and interferon-γ (IFN-γ), are involved in delayed-type hypersensitivity (DTH) reactions, and promote effector responses mediated by CTL, NK cells, and macrophages. T_H2 cells, on the other hand, generate B-cell development, humoral responses, and eosinophilia by their production of IL-4 and IL-5 [19,20]. This distinction recently has been extended to MHC class I-restricted $CD8^+$ T cells. IFN-γ producing type 1 $CD8^+$ cells, with cytotoxic functions, are distinguished from IL-4 producing type 2 $CD8^+$ cells, which suppress antigen-stimulated proliferation of $CD4^+$ T cells [21]. The selection of the T cell subset to be activated and the quality of the ensuing immune response may depend on a range of factors, such as the epitope of the antigen, the type of antigen-presenting cell, the MHC molecules and other accessory signals in antigen presentation, and the past immunologic history of the host as was demonstrated in T cell-mediated responses to infectious

pathogens [20,21].

IL-2 mediates its effect locally, in a paracrine or autocrine fashion, by binding to IL-2-specific receptors. The IL-2 receptor encompasses three transmembrane protein chains. The 55 kD α chain binds IL-2 with very rapid on/off rates, but with a low affinity (affinity constant $Kd=10^{-8}$ M). The association of the 75 kD β chain and the 64 kD γ chain binds IL-2 with intermediate affinity ($Kd=10^{-9}$ M), and is responsible for signal transduction to the cell by its large cytoplasmatic domain. The expression of all three chains results in the formation of the high-affinity heterotrimeric receptor, which binds IL-2 with a 100-fold higher affinity ($Kd=10^{-11}$ M) compared with the βγ chain complex [22,23]. T cells and B cells express high-affinity IL-2 receptors upon antigenic activation. In contrast, the majority of NK cells constitutively express intermediate-affinity IL-2 receptors, and thus are immediately responsive to IL-2. A relatively high concentration of IL-2 is required for saturation of the intermediate-affinity receptor (10^{-8}M *vs.* 10^{-10} M for saturation of the high-affinity heterotrimeric receptor). Activation of immune cells by IL-2 thus depends both on IL-2-receptor expression and IL-2 concentration.

IL-2 and immune effector cells against cancer

Several types of immune cells have been demonstrated to obtain antitumor cytolytic activity when cultured with IL-2. These observations in principle were the background for application of IL-2 *in vivo.*

NK cells comprise 10-15% of peripheral blood mononuclear cells, and proliferate and exhibit enhanced non-MHC-restricted cytolytic activity in response to IL-2 [24,25]. Lymphokine-activated killer (LAK) cells are generated from NK precursors by stimulation of splenocytes or peripheral blood mononuclear cells from normal or tumor-bearing hosts with IL-2 for several days. These LAK cells are capable of lysing a range of fresh and cultured tumor cells but not normal cells *in vitro* [26], and mediate the regression of experimental metastases from murine tumors after adoptive transfer together with IL-2 [27,28]. LAK activity can also be generated *in vivo* by repeated systemic administration of high doses of IL-2 [29].

Monocytes, like NK cells, constitutively express the intermediate-affinity IL-2 receptor [30]. Activation by IL-2 enables monocytes to lyse tumor cells *in vitro* [31].

TIL are observed in many experimental and human solid tumors. IL-2 enables expansion of these TIL *in vitro.* TIL from murine tumors mostly are $CD8^+$ T cells. They have been shown to specifically lyse the autologous tumor *in vitro* [32]. TIL are 50-100 fold more

effective than LAK cells in reducing experimental lung metastases *in vivo* when administered together with IL-2 [33]. This does not prove that direct cytolysis of tumor cells by TIL *in vivo* occurs as also noncytolytic $CD8^+$ TIL mediate the eradication of lung-metastases [34]. Moreover, *in vivo* effectiveness of TIL appears to correlate better with secretion of IFN-γ when cocultured with the autologous tumor than with cytolytic activity *in vitro* [34]. TIL can be grown from human cancers of variable histologic types [35,36] including melanoma [4], renal cell carcinoma [37], lung cancer [6], head and neck cancer [38], and glioblastoma [39]. The majority of these TIL are $CD3^+$ T cells, with variable $CD4^+/CD8^+$ ratios. Freshly isolated human TIL have depressed proliferative [40] and cytotoxic [41,42] responsiveness *in vitro*. This might be due to tumor-derived suppressive factors [11,40]. After expansion with IL-2 TIL often exhibit nonspecific cytolytic activity *in vitro* [36]. Unique cytolytic activity against the autologous tumor has been demonstrated in 30% of human melanomas [4] and rarely in renal cell carcinoma [43]. In melanomas this specific cytotoxicity was proven MHC-restricted [4].

Since recombinant IL-2 became available in 1983 [13] a large number of studies has been performed in cancer patients to evaluate the clinical efficacy of IL-2. These studies were directed towards the induction, proliferation and activation of cytolytic cells, especially LAK cells [26-29]. IL-2 was mostly administered intravenously (i.v.), alone, with LAK cells [44-47], or with IFN-α [48], or subcutaneously (s.c.) [49]. Analogous to cytotoxic chemotherapy, mostly maximally tolerated doses were given, especially in the i.v. trials. The classical schedule described by Rosenberg *et al.* [44] consists of 5-day courses with i.v. IL-2 bolus injections of 100,000 U/kg every eight hours. A dose- and schedule-dependent (rebound) lymphocytosis with enhanced circulating LAK activity occurred [50]. With high-dose IL-2-based treatment schedules objective tumor regression has been obtained in patients with selected tumors. Partial responses (PR) and sometimes durable complete responses (CR) are demonstrated in about 15-30% of patients with malignant melanoma and advanced renal cell carcinoma. Partial responses have also been described in patients with colorectal cancer and non-Hodgkin's lymphoma. Unfortunately, high-dose systemic IL-2 therapy is associated with serious and sometimes life-threatening toxicity, including fever, chills, general malaise, nausea and vomiting, hypotension, fluid retention, respiratory distress and neurologic dysfunction, requiring intensive medical intervention [51]. Adoptive transfer of TIL together with IL-2 is very laborious and expensive, and has been performed in a small number of clinical trials. Objective responses have been described in 25-60% of selected patients with malignant melanoma [52-54] and 30% of patients with renal cell carcinoma [53].

Rationale for low-dose and locoregional IL-2 administration

Most clinical studies on immunotherapy with IL-2 consider high-dose systemic IL-2 administration. This results in objective responses in selected patients only, by a still incompletely understood working mechanism. Yet it is questionable whether high doses of IL-2 on the one hand, and systemic administration on the other hand, are most beneficial to elicit an antitumor response *in vivo*.

Endogenous IL-2 is produced at the site of antigen presentation. It mediates its effect locally at the cell-cell interaction level [14], and its activity normally is not detectable in the human serum [55]. Exogenous IL-2 is rapidly eliminated from the circulation by renal filtration. After i.v. bolus injection a high circulatory peak-level is obtained, which decreases initially with a half-life ($t\alpha$) of 13 min, followed by a slower elimination phase with a half-life ($t\beta$) of 85 min [55]. Due to variable IL-2 receptor affinity, activation of circulating immune cells will depend, among other things, on the obtained serum IL-2 concentration [56]. In the treatment of metastatic murine tumors a biphasic IL-2 dose-response relation was demonstrated, with low-dose therapeutic activity being T cell-mediated, and high-dose activity being mediated by NK or LAK cells [57]. Serum IL-2 concentrations obtained with the common, *i.e.* high dose, clinical i.v. or s.c. treatment schedules are high enough to activate a substantial percentage of the intermediate affinity IL-2 receptors [56,58], as constitutively expressed on NK cells. These treatment schedules are accompanied by serious systemic toxicity. Recently, it was shown that continuous infusion with low doses of IL-2 ($1.5\text{-}4.5\times10^5$ U/m^2/day) is sufficient for selective saturation of high-affinity IL-2 receptors [58]. In patients with lepromatous leprosy such doses of IL-2 applied intradermally generated effective cellular immunity [59,60]. Whether systemic IL-2 administration at the same low doses can elicit an effective antitumor response in cancer patients remains to be proved [61].

Whereas it is doubtful whether TIL and regional lymph-node lymphocytes are affected by systemically administered IL-2, they might be directly activated by locoregionally applied IL-2. Activation of TIL appears preferable above circulating lymphocytes, since TIL may display specific antitumor reactivity *in vitro* [32], and adoptively transfered TIL are far more effective *in vivo* than IL-2-activated peripheral-blood lymphocytes or splenocytes in experimental studies [33]. Relatively low doses of IL-2 will probably be sufficient, which will diminish or avoid systemic toxicity. High local IL-2 concentrations might even be worse. Murine specific CTL clones were shown to develop nonspecific promiscuous lytic ability upon stimulation with high doses IL-2 [62]. Moreover, TIL from immunogenic and nonimmunogenic murine sarcomas, cultured with irradiated autologous tumor in low

levels of IL-2 (10 U/ml), showed increased *in vivo* therapeutic efficacy as compared to TIL cultured with high levels of IL-2 (1,000 U/ml) [63]. The most important motive for locoregional application of IL-2 is, however, that this might provide a more physiological stimulation of the immune system than systemic IL-2 administration. The latter aims at activation of cytolytic cells. Locoregional supply of IL-2, *i.e.* at the site of antigen presentation, might favor sensitization of host immune cells to the tumor and act on the induction phase of an immune response. This hypothesis is reinforced by observations in experimental tumor models, which are reviewed in the next sections. In an analogous situation, in patients non-responsive to hepatitis B vaccine, specific immune responsiveness was elicited by local application of IL-2 at the vaccine injection site [64].

Experimental studies

The antitumor effects and mechanisms of peri- or intratumorally applied IL-2 have been studied in several animal tumors. Preferably syngeneic tumor models were used, because they provide hosts with an intact immune system. The following topics will be reviewed separately: 1. suppression of tumor-growth induced by peritumoral IL-2 injections started shortly after challenge; 2. immune reactions elicited *in vivo* by tumor cells transfected with the IL-2 gene; and 3. locoregional IL-2 therapy of established, macroscopic disease.

Tumor-growth suppression by peritumoral IL-2 administration

Several investigators have demonstrated that inhibition of tumor growth can be obtained by repeated peritumoral IL-2 injections started 0-3 days after s.c. tumor challenge [65-69]. Vaage [68] evaluated the strength of this intervention in a series of mammary carcinomas in syngeneic C3H/He mice. He found that the response to peritumoral IL-2 injections was related to tumor immunogenicity, infiltration of mononuclear cells, and a limited growth rate of the tumor [70]. In the immunogenic MC2 tumor, complete suppression of local tumor growth was obtained in 40-80% of animals with 10-12 daily peritumoral injections of 1,000-5,000 U IL-2 [68,70,71]. Furthermore, Vaage showed that peritumorally administered IL-2 in this tumor not only elicited a local, but also a systemic antitumor reaction, since the growth of uninjected MC2 tumors simultaneously growing on the other flank was suppressed as well [68,70].

Forni *et al.* [65] used the poorly immunogenic methylcholanthrene-induced CE-2 sarcoma in syngeneic BALB/c mice. They described how daily very low-dose (10-20 U) peritumoral

IL-2 injections for 10 days caused complete tumor-growth suppression in 80-100% of animals. A prerequisite, however, was that the subcutaneous tumor challenge was admixed at 1:5 cell ratio with spleen lymphocytes from tumor-bearing mice. These lymphocytes themselves were nonreactive against CE-2 *in vitro* or *in vivo*. This approach showed the same results in several other murine tumors. Moreover, the combination of IL-2 and lymphocytes at the tumor site induced systemic antitumor immunity. Cured mice showed specific DTH reactions and rejected CE-2 rechallenges. Development of this reactivity depended on an intact host immune system, and involved NK cells and T helper cells. It was thought that the lymphocytes inoculated with the tumor cells acted as initiator cells which, after activation with exogenous IL-2, produced cytokines and recruited specific and nonspecific host immune effector mechanisms [72]. Production of IFN-γ played an essential role in the antitumor effect, as also was shown by Vaage in his model [73].

Sigali and Schaefer [66] studied the effects of peritumoral IL-2 injections in the nonimmunogenic B16 melanoma, inoculated s.c. at a lethal dose in syngeneic C57BL/6 mice. Repeated perilesional injections with 30,000 U IL-2, once daily, cured 65% of mice. In this model cured mice did not reject a tumor rechallenge.

Dubinett *et al.* [69] investigated local IL-2 therapy in the poorly immunogenic L1 alveolar carcinoma, syngeneic to BALB/c mice. Twice daily peritumoral IL-2 injections (50,000 U), started on day 3 following s.c. inoculation, and given for 3 weeks, resulted in significant tumor-growth inhibition and increased survival, with a cure rate of 24%. Like in Forni's model [72] both T cell-mediated and nonspecific immune mechanisms appeared to be involved in this therapeutic effect. In this study it was also shown that local IL-2 administration enhanced nonspecific cytolytic activity of both TIL and splenocytes [69].

These studies have in common that the peritumoral administration route was essential for the obtained effect. When equal doses of IL-2 were given i.v. or i.p., tumor-growth inhibition was minor [66] or absent [65,69,73]. In Vaage's model the effect was already lost when IL-2 injections were given 2 cm away from the tumor [70]. Furthermore, low doses of IL-2 were most effective, since a 10- to 20-fold increment of dose did not enhance [72], or even diminished [68] the effect. Only Dubinett *et al.* [69] needed high doses for optimal result.

The use of unmodified IL-2 required repeated injections at short intervals [65,71]. In other studies it was shown that locoregional application of sustained-release formulations such as IL-2 suspended in agar gel [74], entrapped in liposomes [74-76], or coupled to polystyrene beads [77], resulted in equal or increased antitumor activity at less frequent administration compared to unmodified IL-2.

Tumor cells transfected with IL-2 cDNA

The effect of continuous delivery of IL-2 at the tumor site was studied using mouse and rat tumor cell lines in which the gene encoding for IL-2 was inserted with retroviral gene transfer methods [78-83]. These engineered cells constitutively secreted various amounts of IL-2 *in vitro*. They were especially developed to investigate whether the failure of tumors to elicit an effective immune response *in vivo* could be overcome by paracrine stimulation of the host antitumor response. The amount of IL-2 produced by these cells *in vivo* is hard to deduce from the *in vitro* characteristics, and proved to decrease in the only study where this was checked [80]. Nevertheless, after s.c. inoculation in syngeneic mice, the IL-2-producing tumors showed reduced or abrogated tumorigenicity as compared to the more or less immunogenic [78-81] or nonimmunogenic [82,83] parental tumors. This growth-inhibiting effect was extended to admixed unmodified parental cells [79-81,83]. It was shown that the antitumor effect was caused by an immune response induced by the IL-2-producing tumor, which involved $CD8^+$ T cells and NK cells [78,82,83]. The power of the induced immune response depended on the level of IL-2 production as measured *in vitro* [78-80,82,83] and, considering the various studies, apparently on the immunogenicity of the parental tumor. In some models the IL-2-producing tumors induced systemic tumor-specific immunity, thereby protecting the animals against subsequent rechallenge with the parental tumor [78,79,81,82]. Whereas $CD4^+$ T cells were not required for the initial tumor-growth inhibition [78,82,83], they played an essential role in the induction of this immune memory [82].

Histologic examination of the s.c. tumor-challenge site, either of IL-2-producing tumors or following peritumoral IL-2 injections, generally revealed enhancement of the naturally occurring lymphocytic infiltrate. Increased numbers of macrophages [78,84], neutrophilic and eosinophilic granulocytes [72,82], and fibroblasts [83] were also observed. Vaage described an increased fibrotic reaction, encapsulating the tumor [84].

These results support the concept that the host immune response to poorly or nonimmunogenic tumors can be enhanced by protracted local supply of IL-2. It was suggested that the inability of the parental tumors to generate an effective immune response is partly due to failure of T cell help, which is bypassed by local IL-2 supply [78]. The technique of IL-2 gene transfection might be especially important for the development of new tumor vaccines [85].

Regression of grossly established tumors induced by local IL-2 application

The therapeutic effects of locoregional IL-2 immunotherapy in macroscopic disease have been studied in s.c. [68,86], i.p. [87] and metastatic [76] syngeneic tumor models, and in

a spontaneous bovine tumor [88].

Regression of large tumor masses was observed by Maas *et al.* [87] in the weakly immunogenic SL2 lymphoma, i.p. inoculated in syngeneic DBA/2 mice. Whereas untreated animals developed large tumor burdens and died within 20 days, 70% of the animals receiving i.p. injections with IL-2 on days 10-14 were cured. Systemic antitumor activity developed, since 50% of simultaneously inoculated s.c. SL2 tumors were cured as well [87]. Furthermore, surviving animals rejected subsequent tumor rechallenges [89]. This systemic reaction was found to be a tumor-specific T cell-mediated effect. Both the IL-2 application site and the time schedule proved to be critical in this model. I.p. IL-2 injections in animals without i.p. tumor only had marginal effects on s.c. growing tumors. Furthermore, IL-2 injections in an earlier phase of i.p. tumor development (day 3-7) had no therapeutic effect [89]. It was suggested that IL-2 reinforces a specific immune response which takes about a week to develop. The same phenomenon was observed by others in the immunogenic murine myeloma X5563, which could be cured by repeated local IL-2 injections started on day 7, but not on day 1 after intradermal inoculation [86]. However, in the earlier mentioned immunogenic MC2 mammary carcinoma, tumor growth was suppressed completely with peritumoral IL-2 injections at 1,000 U started 1 day after implantation, and cure was still obtained when IL-2 injections at 5,000 U were started in palpable 9 day-old tumors [68].

Anderson *et al.* [76] studied the antitumor effects of intrathoracic IL-2 injections on 5 day-old experimentally induced pulmonary metastases of the weakly immunogenic MCA-106 sarcoma in C57BL/6 mice. The number of lung metastases was significantly decreased by 3-5 daily intrathoracic IL-2 injections, whereas i.v. or i.p. administration had no effect.

In a quite different animal, Rutten *et al.* [88] confirmed the potential therapeutic effects of local IL-2 therapy in established tumors. In 5 cows with spontaneous bovine ocular squamous-cell carcinoma, ranging from 1-4 cm in diameter, 10 combined intratumoral and perilymphatic injections with IL-2 resulted in 3 CR and 1 PR. A remarkable protracted tumor-regression time, ranging from 2-7 months, was observed in this study.

These experimental studies show that locoregional injections with IL-2 alone can induce complete regression of palpable tumor masses. The daily dose of IL-2 in all these studies varied from 5,000-50,000 U, and therapy was well tolerated. The prevalence of peritumoral injection over systemic administration in established tumors was demonstrated in 2 models [76,89], and is also suggested when these experimental studies are compared to those of Rosenberg and colleagues using systemic IL-2 treatment. These investigators aimed at the generation of LAK activity *in vivo* by three times daily i.p. IL-2 administration [27,28,90]. The effects on s.c. growing tumors was studied using the weakly

immunogenic MCA 105 sarcoma. Complete regression of 3-10 day old tumors was obtained in 20-50% of animals at very high doses of IL-2 only (500,000-600,000 U/day i.p.), which caused severe toxicity [90]. Most experiments with systemic IL-2 treatment were directed towards 3-10 day old experimental liver and lung metastases of syngeneic murine tumors. These metastases were inhibited, but not cured by high doses (60,000-300,000 U/day) of IL-2 alone [27,90]. The antitumor effect in these studies was related to the immunogenicity of the tumor [90]. It was LAK-effector mediated, but in immunogenic tumors also T cells were involved [28]. The efficacy of this treatment was enhanced when combined with i.v. LAK cell administration [27,90].

Clinical studies with locoregional IL-2 administration

The therapeutical effects of locoregionally administered IL-2 in human cancer have been studied on a modest scale. Most clinical studies concern local recurrent or residual disease, or primary locally far-advanced disease for which no conventional treatment was available. IL-2 was given alone or combined with LAK cells. Access to the tumor was obtained by direct, endoscopic or ultrasound-guided intra- or peritumoral injection, perilymphatic injection, *i.e.* around the tumor-draining lymph nodes, selected intra-arterial infusion, or compartmental administration into localized body cavities such as the peritoneum, pleura, bladder, or leptomeninges. Especially in the studies with intracavitary IL-2 administration high doses were used, aiming at high and prolonged IL-2 levels sufficient to generate or maintain LAK activity. Published studies on locoregional IL-2 administration will be discussed by tumor site. Details are summarized in Table 1.

Head and neck squamous-cell carcinoma (HNSCC) is characterized by locoregional tumor spread near extensive submucosal and nodular lymphatic tissue. The first therapeutical study with IL-2 in this disease was based on Forni's experimental results [65]. In this study Cortesina *et al.* [91] gave patients with locoregionally recurrent HNSCC perilymphatic injections with low doses IL-2 in order to activate regional lymphocytes. They obtained 3 CR (duration 4-6 months) and 3 PR in 10 patients. Subsequent studies using perilymphatic treatment schedules did not equal this high response rate [92-95]. Responses were also described with peritumoral [96] and intra-arterial [97,98] IL-2 administration. Generally, fewer responses were seen in patients with large tumor masses. In perilymphatic treatment responses appeared unlikely in patients with bilateral lymph-node dissection [91]. No conclusions can be drawn on the most beneficial IL-2 dose or route. In the 3 dose-escalation studies the objective responses occurred at the lowest IL-2 dose used [97],

at the highest [95], or both [94]. Systemic toxicity varied from none [91-93] or mild fever only [95,96,98], to significant at high-dose levels in the dose-escalation studies [94,97]. Temporary moderate local swelling, redness, or pain appeared not to be dose related [91,95,97]. Histological examination of treated tumors showed increased lymphocytic infiltration, macrophages, giant cells, and fibrous changes at the tumor site, and degeneration of cancer cells [96,98].

In bladder carcinoma, immunotherapy has a longer history, and the intravesical application of the microbial immunostimulant bacillus Calmette-Guérin (BCG) has been proven effective in superficial tumors [99]. Pathological CR and PR were also described using intratumoral, intravesical or intra-arterial IL-2 administration [100-103]. As in HNSCC the occurrence of systemic adverse effects depended on the dose used. Immunohistochemical analysis of treated tumors revealed increased numbers of infiltrating activated T cells and macrophages [104].

In patients with recurrent or residual brain tumors, local adoptive immunotherapy with IL-2 and LAK cells has been applied [105-107]. Few tumor regressions were found. Systemic side effects including fever and chills, and neurological side effects related to increased cerebral edema [106,107] occurred in most patients. Increased intracranial pressure was also described in 2 case reports on high-dose intraventricular IL-2 immunotherapy for leptomeningeal melanoma metastases [108,109]. After intraventricular injection IL-2 is cleared from the cerebrospinal fluid with a half-life of 4-8 h [110]. Treatment-induced secondary cytokines, soluble IL-2 receptor and neutrophilic leukocytes, sustained presence of lymphocytes, and decrease of malignant cells were shown in the cerebrospinal fluid [110].

Ultrasound-guided intratumoral IL-2 administration was described in few patients with small hepatocellular carcinomas (<3.5 cm) [111,112]. One PR was recorded. Transient fever and chills occurred. Repeated tumor biopsies from patients with stable disease (SD; duration 3-8 months) showed decreased numbers of neoplastic cells, tumor necrosis, fibrosis, and infiltration of lymphocytes and granulocytes [112].

Intrapleural administration of IL-2 was studied in patients with malignant pleural effusion. In one study it was shown that intrapleural injection with 1,000 U of IL-2 resulted in a local level of IL-2 gradually decreasing from 28 U/ml at 30 min to 4 U/ml at 24 h, while serum levels of IL-2 were <0.01 U/ml [113]. Increased numbers of intrapleural lymphocytes and eosinophils, disappearance of malignant cells, and subsequent disappearance of the pleural effusion occurred after repeated injections in 9 out of 11 patients, enabling surgical treatment of the primary tumor in 6 [113]. Objective tumor regression was

Table 1. *Clinical studies with locoregional IL-2 immunotherapy.*

Tumor, Reference	Treatment with IL-2			Patients	
	Route	Schedule[a]	Daily dose ($\times 10^3$ U)	n	Response
HNSCC					
Cortesina *et al.* [91]	perilymphatic inj.	10 days	0.2[b]	10	3 CR, 3 PR
Cortesina *et al.* [92]	perilymphatic inj.	10 days	1-5	10	7 MR[c]
Mattijssen *et al.* [93]	perilymphatic inj.	10 days	0.2-0.4	15	none
Vlock *et al.* [94]	perilymphatic + peritumoral inj.	2×5 days	0.2-4,000	36	2 PR
Squadrelli *et al.* [95]	perilymphatic inj. + peritumoral LAK cells	10 days	2.4-1,800	14	3 PR, 3 MR
Saito *et al.* [96]	peritumoral inj.	9-38 days	800	20	2CR,1PR,1MR
Gore *et al.* [97]	cont. intra-arterial inf.	5-10 days	30-30,000	12	2 PR
Ishikawa *et al.* [98]	intra-arterial inf. + LAK cells	4-15 times	1	6	6 regression, not quantified
Bladder carcinoma					
Pizza *et al.* [100]	intratumoral inj.	2-3 times	2-4[b]	10	3 CR, 2 PR
Fujioka *et al.* [101]	intratumoral inj.	3 days	500	12	1 CR, 2 PR
Huland *et al.* [102]	cont. bladder perfusion	5 days	3,000[b]	5	1 CR
Tubaro *et al.* [103]	cont. intra-arterial inf.	2×5 days	18-18,000/m^2	12	2 CR, 3 PR
Malignant brain tumors					
Yoshida *et al.* [105]	intratumoral inj. + LAK cells	2-3 days/wk	0.05-0.4	23	6 regression, not quantified
Barba *et al.* [106]	intratumoral inj. + LAK cells	5 days	10-60/kg	9	1 PR
Merchant *et al.* [107]	intra- or peritumoral inj. + LAK cells	4 days	1,000	13	none
Leptomeningeal metastases					
Melanoma, lung carcinoma:					
List *et al.* [110]	intraventricular inj.	2 days/wk	1,000-12,000	11	8 responses[d]
Hepatocellular carcinoma					
Shirai *et al.* [111]	intratumoral inj.	2-4 times	450-900	5	1 PR, 1 MR
Gandolfi *et al.* [112]	intratumoral inj. + LAK cells	3-20 times	10-30	5	none

Table 1, continued

Tumor, Reference	Treatment with IL-2 Route	Schedule[a]	Daily dose ($\times 10^3$ U)	Patients n	Response
Malignant pleural effusion					
Lung carcinoma:					
Yasumoto *et al.* [113]	intrapleural inj.	10-28 days	1	11	9 effusion disappeared
Mesothelioma, carcinomas:					
Astoul *et al.* [114]	cont. intrapleural inf.	5 days	3,000-24,000	22	1 CR, 9 PR
Intraperitoneal malignancies					
Ovarian, colon carcinoma:					
Steis *et al.* [115]	intraperitoneal inj. + LAK cells	5 days	75/kg	24	7 PR

inj., injection; inf., infusion; cont., continuous.

[a] Indicated are either the duration of 1 treatment course (in days; daily treatment; mostly 1-2 courses were given) or the total number of administrations (times).

[b] Natural IL-2 was used

[c] 6 additional responses recorded in 10 additional patients are identical to the previous publication [91].

[d] Defined as complete clearing of cerebrospinal fluid tumor on 2 sequential weekly lumbar punctures.

described in patients with pleural carcinoma metastases and early-stage mesotheliomas [114]. Fever and transient increase of pleural effusion were recorded in both studies, while serious systemic toxicity occurred at the highest dose levels only.

Intraperitoneal immunotherapy with high doses IL-2 and LAK cells has been applied in patients with malignancies limited to the peritoneal space, such as ovarian and colorectal cancer. Laparoscopy- or laparotomy-documented PR were obtained [115]. A special and major problem encountered with this approach was intraperitoneal fibrosis, limiting repeated IL-2 administration. Systemic side effects were similar to those reported in i.v. high-dose IL-2 treatment.

Conclusion

In several experimental tumor models it has been shown that repeated or sustained supply of low doses IL-2 directly at the tumor site can elicit a systemic antitumor immune response in poorly immunogenic tumors. Both specific T cells and nonspecific immune mechanisms appear to be involved. The induced immune response mediates inhibition or regression of local and distant tumor sites. In this respect, local IL-2 administration is clearly superior to systemic IL-2 therapy. Whether local IL-2 supply is beneficial in human cancer remains to be proved. While several thousands of patients have been treated with high-dose systemic IL-2 therapy, there is only limited clinical experience with locoregional IL-2 application. Since various schedules, doses, and administration routes have been used in a variety of clinical situations, it is difficult to draw conclusions. Objective local tumor responses have been described especially in head and neck cancer and bladder cancer. The required access to the tumor might limit the application of locoregional IL-2 therapy. On the other hand, the absence of serious toxicity is advantageous. Clinical responses are unlikely to occur in patients with large tumor masses. This might be related to depressed host immune responsiveness. The recently described structural and functional alterations in the T cell antigen receptor of advanced tumor-bearing mice and cancer patients might be the basis for depressed T cell responsiveness [116]. The future challenge will be to define the conditions and the mechanisms of beneficial effects of local IL-2 administration.

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Chapter 3

Determination of HLA antigen expression in routinely processed head and neck squamous-cell carcinoma lesions

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Summary

Background: Since $CD8^+$ and $CD4^+$ T lymphocytes recognize antigens only in combination with, respectively, class I and class II human leukocyte antigens (HLA), the latter are supposed to play an important role in host-tumor interactions. Because of that we explored the expression of HLA class I and II antigens by head and neck squamous-cell carcinomas (HNSCC).

Methods: Paraffin-embedded samples from 66 HNSCC and 7 verrucous carcinomas were studied immunohistochemically using anti-HLA class I monoclonal antibodies (MAbs) HC10 and HCA2, and anti-HLA-DR rabbit serum. Percent stained tumor cells were scored in 1 of 5 categories. The scores of 40 tumors were compared to the staining results obtained on frozen sections of the corresponding lesions, including those of the anti-class I MAb W6/32.

Results: High percentage-matched scores for paraffin and frozen sections were obtained, with HC10 *vs.* HC10, HC10 *vs.* W6/32, and anti-HLA-DR *vs.* anti-HLA-DR showing the best correlations. HLA class I expression in HNSCC was high (*i.e.* in 49/66 lesions >50% cells were stained), and correlated with the degree of differentiation, and inversely with the modified Jakobsson score (*i.e.* high expression was observed in well and moderately differentiated carcinomas). HLA class II expression (>5% cells stained) was found in 21/66 HNSCCs and correlated inversely with the degree of differentiation. From 4 HNSCC subsites, oral cavity carcinomas showed the highest HLA class I expression.

Conclusions: Routinely processed paraffin-embedded tumor samples are sufficient for immunohistochemical detection of HLA class I and II expression. In HNSCC a high level of HLA class I expression was observed especially in tumors from the oral cavity, which suggests susceptibility to $CD8^+$ T cells.

Introduction

HLA antigens are supposed to play a role in the host's immune response towards neoplastic cells. On a variety of cancer cells both defective HLA class I and *de novo* class II expression have been described, mostly using immunohistochemistry on frozen tissue samples [1]. Low expression of HLA class I antigens correlates with poor differentiation in colorectal cancer [2] and in larynx carcinoma [3], and with poor prognosis in patients with locoregional melanoma metastases [4]. HLA class II expression in primary melanoma is associated with shorter disease-free survival [5]. The significance of HLA expression on tumor cells in relation to immunotherapy is mainly speculative, and has seldom been investigated. High HLA class II expression in regressing melanoma lesions following recombinant interleukin-2 (IL-2)-based immunotherapy has been described [6].

Immunohistochemical analysis of HLA antigen expression is largely restricted to fresh frozen tissue. Routine tissue processing, *i.e.*, formaldehyde-fixation and paraffin-embedding, denatures the antigens, and as a result the reactivity of most antibodies is lost. HCA2 and HC10 MAbs, however, were prepared against free denatured HLA-A and -B locus heavy chains, respectively, and found to be reactive in paraffin sections of normal tissues [7]. For detecting HLA class II antigen expression in frozen and paraffin sections rabbit polyclonal anti-class II sera have been developed (H.L. Ploegh, Amsterdam).

Our first aim was to evaluate the immunohistochemical detection of HLA antigen expression on tumor cells using these antibodies on routinely processed tissue, by comparing results on frozen and paraffin sections of the same tumor. Such detection method proved feasible on paraffin sections of HNSCC. The sample group was thus enlarged to include the distribution of HLA antigens in HNSCC in relation to primary site, stage of disease and histological grade. We are at present studying local IL-2 immunotherapy as a new treatment approach for HNSCC [8]. Answering these questions might be of relevance for the design and interpretation of such clinical studies.

Materials and methods

Patients and tumor specimens

Routinely processed paraffin-embedded tissue samples of the primary tumor from 73 patients with squamous-cell carcinoma (SCC, n=66) or verrucous carcinoma (n=7) of the head and neck were used. Eighteen samples were decalcified according to standard procedures. The samples were taken either at surgical tumor excision (n=50) or from

tumor biopsies (n=23). At the same time, a fresh tumor sample from 40 of the patients was also deep frozen and stored into liquid nitrogen until use.

The SCC patients were selected to compose 4 site-specific groups (oral cavity, oropharynx, hypopharynx, and larynx) which were matched for stage of disease and histological grade (Table 1). All patients came from the Departments of Otorhinolaryngology and Maxillo-Facial Surgery, University Hospital Nijmegen, between 1974 and 1990. The group consisted of 58 men and 15 women, with a mean age of 61 years (range 33-81 years). They were not previously treated, except for 4 patients who had prior radiotherapy. The tumors were staged according to the TNM classification and scored in stages I to IV according to UICC (1987).

The pathological diagnosis and grading was assessed on the paraffin sections. All tumors were scored according to the modified Jakobsson system for SCC [9]. In this semi-quantitative grading scheme, 6 parameters on the tumor cell population and the tumor-host interface are scored in a 1 to 4 point scale.

Table 1. *Clinical and histopathological characteristics of the head and neck squamous-cell carcinomas according to site*

		T (TNM)[a]		Stage[a]		Histopathological grade				Jakobsson score[c]		
Site	n	1+2	3+4	I+II	III+IV	well	mod.	poor	undif.[b]	≤12	13-15	≥16
Oral cavity	19	10	8	6	12	1	15	3	0	9	6	3
Oropharynx	15	5	9	4	10	0	11	3	1	6	5	3
Hypopharynx	7	1	6	1	6	0	4	3	0	1	3	3
Larynx	25	12	11	9	14	1	16	7	1	9	11	5
Total	66	28	34	20	42	2	46	16	2	25	25	14

[a] Four tumors represent recurrent disease, and were not classified.

[b] Well, moderately, and poorly differentiated squamous-cell carcinomas and undifferentiated carcinomas.

[c] Two cases were not scored because tumor surrounding tissue lacked in the samples.

Table 2. *Antibodies used for immunohistochemical analysis of HLA antigen expression on tumor cells.*

Antibody	Specificity	Dilutions frozen sect.	Dilutions paraff. sect.	Source	Ref.
MAb W6/32	HLA-A,B,C heavy chains, β2-microglobulin bound	1:5000 (2-step)	not applicable	Seralab	[14]
MAb HC10	HLA-B,C heavy chains	1:2500 (2-step)	1:400 (2-step)	H.Ploegh	[15]
MAb HCA2	HLA-A heavy chains	1:80 (3-step)	1:80 (2-step)	H.Ploegh	[7]
RaHC rabbit serum	HLA-A,B,C heavy chains	not done	1:80 (3-step)	H.Ploegh	[15]
DR rabbit serum	HLA class II, DR mainly	1:1200 (2-step)	1:40 (3-step)	H.Ploegh	personal communic.

Immunohistochemistry

To evaluate HLA antigen expression on tumor cells a 2- or 3-step immunoperoxidase procedure on 4 μm thick frozen or paraffin sections was performed. The antibodies used, their specificity, dilution, and source are summarized in Table 2. Beside the newly developed anti-class I MAbs HC10 and HCA2, the W6/32 MAb was used on frozen sections as a standard, and the rabbit RaHC serum, reactive with all 3 class I heavy chains, was added to evaluate the paraffin sections. The anti-HLA-DR serum used was raised in rabbits against intact purified HLA-DR.

Cryostat sections were air-dried and fixed in 100% acetone. For staining with the polyclonal anti-HLA-DR serum sections were pre-incubated with 20% normal swine serum (NSwS) for 10 min to prevent non-specific binding of the secondary antibody to the tissue. For paraffin sections the procedure was as follows: they were deparaffinized with xylene, hydrated in graded alcohols, and immersed in 3% H_2O_2 in phosphate buffered saline (PBS) for 30 min to block endogenous peroxidase activity. Then they were pre-incubated with 20% normal rabbit serum (for staining with HC10 and HCA2), or with 20% NSwS (for RaHC), or 100% NSwS (for anti-HLA-DR). Cryostat or paraffin sections were incubated with the primary antibody for 60 min. This was followed by incubation with the second, and in case of a 3-step method also with the third peroxidase labeled antibody for 30 min each. Between all steps the slides were washed in PBS. The peroxidase label was visualized by incubation with a 3,3' diaminobenzidine tetrahydrochloride solution as a substrate for 5 min. Sections were counterstained with Harris hematoxylin, dehydrated and mounted. The whole procedure was performed at

room temperature. From all samples negative controls were obtained by skipping incubation with the primary antibody.

Evaluation

All slides were read by 2 observers. The staining intensity of the tumor cells was scored as negative (-), weak (+), moderate (++) or strong (+++). Expression of antigens on the tumor cells was estimated as a percentage of the total number of tumor cells in the slide, and scored in one of the following categories: 0-5%; 6-25%; 26-50%; 51-75%; and 76-100%. Necrotic or keratinized areas of tumor tissue were not taken into consideration. Well differentiated keratinizing tumors, however, were scored in the highest category if the basal non-keratinizing layers were completely positive.

Statistical analysis

Correlations between staining results on paraffin and frozen sections from the same tumors were assessed by calculating kappa coefficients [10]. This is a parameter of agreement, which takes chance agreement into account. A kappa value of 0 represents only chance agreement, and the more kappa approximates 1, the better the agreement. Staining results of decalcified and non-decalcified paraffin sections were compared with the Wilcoxon rank test. Non-parametric tests (Wilcoxon rank test and Kruskal-Wallis test) were used to find possible differences in clinico-pathological parameters for the 4 site-specific groups. Correlations between tumor HLA antigen expression and clinical and pathological parameters were evaluated by calculating Pearson or Spearman correlation coefficients.

Results

Quality of staining

Staining properties will be given for each antibody. The W6/32 MAb showed very strong and homogeneous staining with almost all tumors. In several sections normal mucosa was present, which always revealed HLA class I expression, especially in the basal layers. The HC10 MAb gave moderate to strong intensity in most paraffin and frozen sections. Heterogeneous expression of HLA class I was observed in several tumors. HC10, like W6/32, showed mainly membranous, but also cytoplasmatic HLA class I expression on tumor cells (Figs. 1 and 2). The staining patterns of the RaHC serum resembled very much those of HC10, however the intensity was markedly lower, and the number of tumor cells stained with RaHC was equal or lower than with HC10. The HCA2 MAb

exhibited a homogeneous, diffuse cytoplasmatic staining pattern which was less clear than with HC10. Some non-specific background staining was seen.

The staining intensity of the anti-HLA-DR serum was moderate in both paraffin and frozen sections. In the lymphatic tissue surrounding some tumors a typical follicular HLA class II distribution was seen. In several tumors a heterogeneous, mainly cytoplasmatic expression of HLA class II antigens was observed (Fig. 3).

Decalcification of paraffin sections did not influence the quality of staining, nor the scoring. This was confirmed by comparing statistically the results on decalcified and non-decalcified sections.

Comparison of results in frozen and paraffin sections of the same tumor

These results are based on 40 tumors from which both paraffin and frozen sections were available. The percentage positive tumor cells was scored in 1 of 5 categories as described above. Comparing the results of 2 slides, scores in the same and also in an adjacent category were considered acceptable. In this way, percentage-matched scores for paraffin and frozen sections were calculated, and found to be high (>80%). The kappa correlations indicate fair to good levels of agreement for HC10 as compared to HC10, and for anti-HLA-DR as compared to anti-HLA-DR (Table 3).

Table 3. *Immunohistochemically detected HLA antigen expression on tumor cells: comparison of result in paraffin (par) and frozen (fr) sections of the same tumor (n=40).*

Antibodies/sections	% match[a]	kappa[a]	p^a	% match[b]	kappa[b]	p^b
HC10 par *vs.* W6/32 fr	51	0.15	0.04	81	0.35	0.004
HCA2 par *vs.* W6/32 fr	68	0.10	n.s.	92	0.09	n.s.
HC10 par *vs.* HC10 fr	50	0.30	<0.001	83	0.56	<0.001
HCA2 par *vs.* HCA2 fr	70	0.27	0.005	90	0.35	0.007
HC10 par *vs.* RaHC par	40	0.22	<0.001	74	0.45	<0.0001
HLA-DR par *vs.* HLA-DR fr	60	0.29	0.002	90	0.60	<0.001

The estimated percentage stained tumor cells in each slide is scored in 1 of 5 categories.

[a] Scores in the same category accepted.

[b] Scores in the same or an adjacent category accepted.

n.s., not significant.

Table 4. *HLA antigen expression on tumor cells in primary head and neck squamous-cell carcinomas of different sites.*

		HLA class I[a]					HLA class II[b]				
Site	n	≤5	6-25	26-50	51-75	76-100	≤5	6-25	26-50	51-75	76-100[c]
Oral cavity	19	0	1	0	6	12	12	4	1	2	0
Oropharynx	15	0	3	2	3	7	9	3	1	2	0
Hypopharynx	7	0	0	2	4	1	5	2	0	0	0
Larynx	25	2	5	2	5	11	19	3	1	1	1
Total	66	2	9	6	18	31	45	12	3	5	1

Immunohistochemically detected in paraffin sections with MAb HC10[a] and anti-HLA-DR rabbit serum[b]. Percentage tumor cells stained scored in 1 of 5 categories[c].

HLA class I and II expression on SCC

Table 4 contains the results of the evaluation of paraffin sections of 66 squamous-cell carcinomas stained with MAb HC10 and anti-HLA-DR rabbit serum. Overall, HLA class I antigens were expressed on more than 50% of the tumor cells in 49 tumors (74%). Only 2 tumors were completely negative. A moderate but significant correlation was found between the level of HLA class I expression and the degree of differentiation (Pearson corr. coeff. 0.26; p=0.04), and also inversely with the Jakobsson score (Spearman corr. coeff. -0.26; p=0.03), *i.e.*, high class I expression was observed in well and moderately differentiated carcinomas, and in tumors with a low Jakobsson score.

HLA class II antigens were expressed on more than 5% of the tumor cells in 21 tumors (32%), including 9 tumors (14%) with more than 25% tumor cells stained. HLA class II expression was inversely correlated with the degree of differentiation (Pearson corr. coeff. -0.27; p=0.03) *i.e.*, high class II expression was more frequently observed in poorly differentiated carcinomas.

No correlation was found between HLA class I or II expression and TNM classification or stage of disease.

HLA antigen expression on tumors of the 4 different subsites was compared independently of the other parameters, as no significant differences were found for the distribution of the TNM classification, stage, histological grade, and Jakobsson score over these 4 groups (Table 1). The tumors from the oral cavity (mostly oral tongue and floor of the mouth) showed a significantly higher HLA class I expression as compared to the larynx carcinomas (Table 5, Chi square test, p=0.04). No differences in HLA class II expression related to tumor site were found.

Table 5. *HLA class I expression in relation to tumor site.*

		% cells stained[a]	
Site	n	≤50%	>50%
Oral cavity	19	1	18
Larynx	25	9	16

Chi^2 =4.19; df=1; p=0.04.

[a] Immunohistochemically detected in paraffin sections with MAb HC10.

HLA class I and II expression on verrucous carcinoma

Seven verrucous carcinomas were evaluated, 2 from the oral cavity and 5 from the larynx. They had a mean Jakobsson score of 7. All had a very high HLA class I expression (76-100% of the tumor cells stained) as detected with MAb HC10 in paraffin sections (Fig. 4). HLA class II was expressed on >5% of the tumor cells in 5 (71%), and on >25% in 1 tumor (14%).

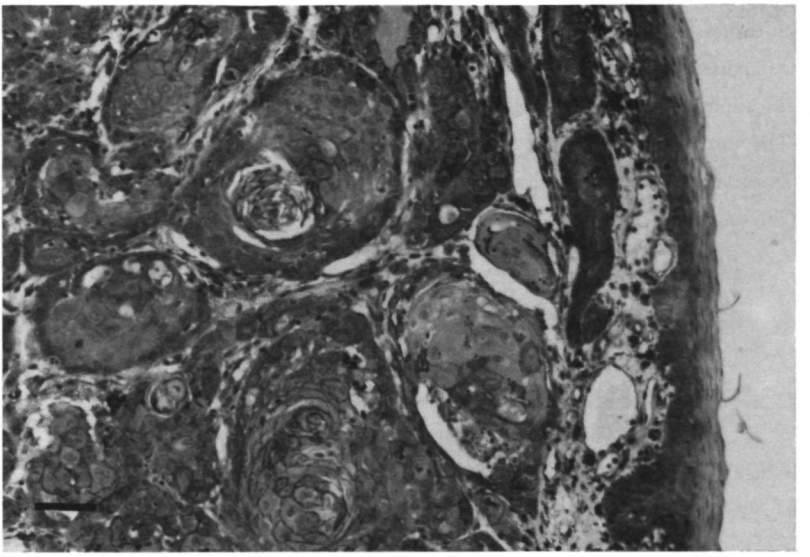

Figure 1. Moderately differentiated SCC of the oral tongue, paraffin section, stained with MAb HC10. Heterogeneous HLA class I expression on the tumor cells is illustrated. The normal mucosa (on the right) showes staining in the basal layers. Bar = 20 μm.

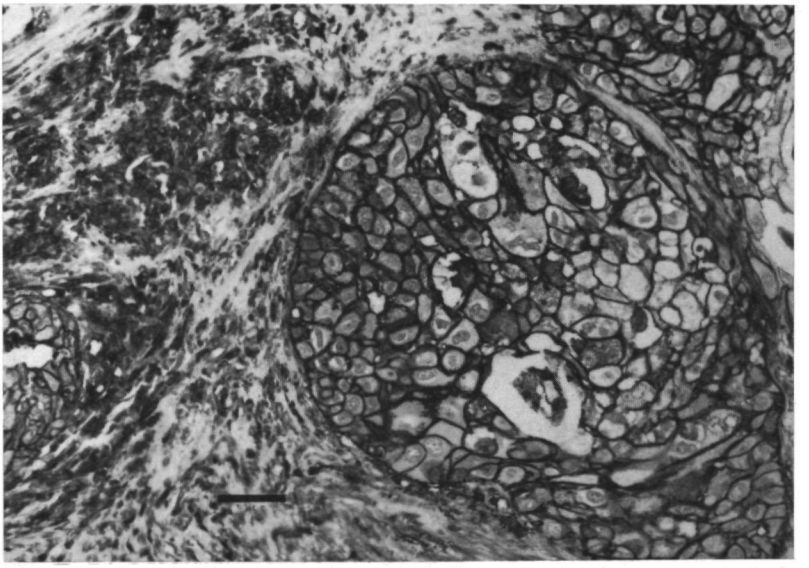

Figure 2. Moderately differentiated SCC of the larynx, paraffin section, stained with MAb HC10, showing homogeneous, mainly membranous staining pattern of tumor cells. Bar = 20 μm.

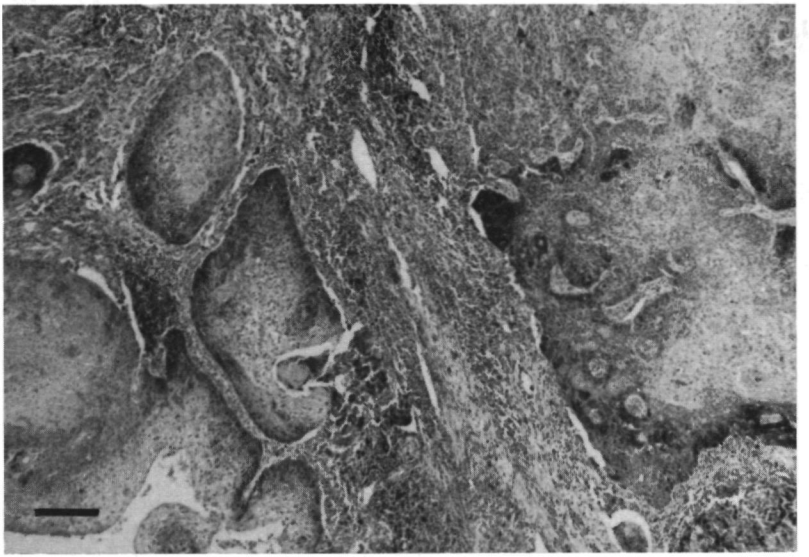

Figure 3. Paraffin section of moderately differentiated SCC of the oropharynx, stained with anti-HLA-DR serum. Staining of tumor cells with heterogeneous intensity is locally seen. Bar = 60 μm.

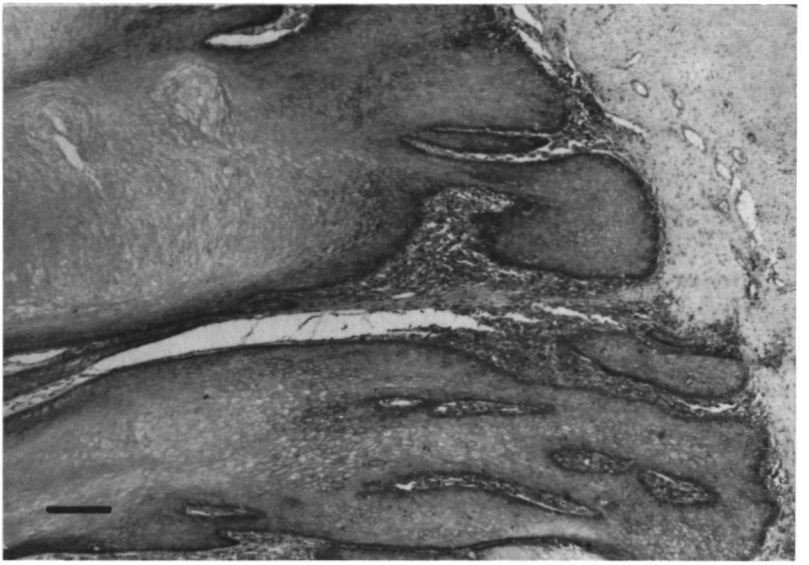

Figure 4. Paraffin section of verrucous carcinoma of the larynx, stained with MAb HC10, scored in the highest category for HLA class I expression. Bar = 60 μm.

Discussion

A panel of recently developed anti-HLA antibodies was tested on paraffin sections of HNSCCs. For HLA class I detection on paraffin sections the HC10 and HCA2 MAbs were compared with the W6/32 MAb on frozen sections of the same tumors. Although a high percentage of matched scores (81% and 92%, respectively) was found, the kappa correlations were moderate or, in case of HCA2 even not significant. Several explanations are possible for these findings. Statistical calculations are highly influenced by the distorted distribution of HLA class I expression detected with W6/32 in this group, which was found in the highest category in almost all cases. Furthermore, W6/32 has a somewhat broader reactivity than other anti-HLA class I MAbs [4]. We performed additional staining with the anti-HLA class I MAbs BRL [4] and B9 [5] in 14 of the 40 frozen tumor sections and found a somewhat lower class I expression as compared to W6/32 as well (results not shown). Besides, W6/32, HC10 and HCA2 are directed against different antigenic determinants of HLA class I. Immunohistochemical staining with the anti-HLA-DR rabbit serum gave quantitatively and qualitatively comparable results on frozen and paraffin sections.

We conclude that immunohistochemical detection of HLA antigen expression with these antibodies on conventionally processed tumor tissue samples is reliable. For HLA class I expression the MAb HC10 is preferred to HCA2 and RaHC because of the best correlation with W6/32 and the clearest staining pattern. Advantages of this method are the much higher availability and the better preserved morphology of paraffin-embedded tissue as compared to frozen material.

We used this technique to evaluate the HLA antigen expression on tumor cells in 66 paraffin-embedded HNSCCs. Overall, high HLA class I antigen expression was found, especially in well and moderately differentiated carcinomas and in tumors with a low Jakobsson score, in agreement with the findings of Esteban *et al.* [3] in frozen sections of 38 larynx and hypopharynx carcinomas. There was no evidence for selective HLA-A or -B loss when comparing the results of RaHC (anti-HLA-A,B,C), HCA2 (anti-HLA-A) and HC10 (anti-HLA-B,C). However, only immunohistochemical and no molecular biological techniques were used.

HLA class II antigen expression on tumor cells was locally observed in a minority of the SCCs. An inverse correlation between class II expression and degree of differentiation has also been found by Houck *et al.* [11], evaluating frozen sections of 30 HNSCCs. In a separately analyzed group of verrucous carcinomas, however, which are a highly differentiated variant of SCC, we found relatively high HLA class II expression, as

compared to the SCC. Although topological correlation between HLA class II-expressing tumor cells and infiltration of lymphocytic cells was observed in some tumors, both verrucous and non-verrucous, this could not consistently explain HLA class II staining patterns. Our results on HLA class II expression do not indicate independent prognostic relevance of this factor, and are not in accord with those of Esteban *et al.* [12], who found far more restricted class II expression in a group of 69 SCCs of the larynx. Differences in results of immunohistochemical studies, however, may be related to differences in techniques, antibodies and lesions used, and interpretation of staining patterns.

Head and neck squamous-cell carcinoma represents a heterogeneous group of diseases. Their biological behavior and prognosis are related to stage of disease and primary tumor site. It thus appears reasonable to compare different subgroups when evaluating possible prognostic parameters. We did not find stage-related differences in HLA class I and II expression. However, clear site-related class I expression was observed, and high HLA class I expression was observed especially in the carcinomas of the oral cavity.

When using local IL-2 immunotherapy in an animal tumor model, the tumor response highly depends on the presence of peritumoral lymphocytes [13]. In this respect, the well developed lymphoid tissue in the oral mucosa may be important. The high HLA class I expression in carcinomas of the oral cavity implies susceptibility to interaction with $CD8^+$ cytotoxic T cells. Tumors of the oral cavity are also easily accessible to local injections and to clinical observation. This suggests that the subgroup of oral cavity carcinomas might be well suited for local immunotherapy among HNSCCs.

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Chapter 4

Perilymphatic administration of recombinant interleukin-2 in patients with locally far-advanced, nonpretreated head and neck squamous-cell carcinoma

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Summary

Background: In experimental tumor models specific and nonspecific host effector mechanisms were recruited by local low-dose interleukin-2 (IL-2) injections and the presence of otherwise nonreacting lymphocytes at the tumor site (*J Immunol* 1985; 134:1305). This approach was applied in head and neck squamous-cell carcinoma patients (HNSCC), and the first study reported 6 objective tumor responses in 10 patients (*Cancer* 1988; 62:2482).

Patients and methods: In 15 patients with locally far-advanced, nonpretreated HNSCC daily low-dose (200-4,000 U) perilymphatic IL-2 injections were given for 10 days. Tumor biopsies were taken before and after treatment for histopathological evaluation of the mononuclear cell infiltrate and immunohistochemical detection of human leukocyte antigen (HLA) expression on tumor cells.

Results: No adverse effects were recorded. There were no objective responses. HLA class I and II antigen expression was detectable in 9/10 and in 2/10 tumors, respectively. As compared to pretreatment biopsies, no changes in the mononuclear cell infiltrate or in HLA expression were found following treatment.

Conclusions: Low-dose perilymphatic IL-2 therapy is not effective in locally far-advanced HNSCC. The discrepancy with the previously published clinical study might be related to tumor size.

Introduction

The potential of systemically administered interleukin-2 (IL-2) in cancer immunotherapy is presently being explored in many centers. High doses of IL-2, alone or in combination with lymphokine-activated killer cells, induce tumor regression in a number of patients with metastasized melanoma and renal cell carcinoma [1]. However, this treatment is accompanied by serious toxicity. Locoregional administration of IL-2, on the other hand, has had less attention, but may have important advantages in certain tumor types [2].

Forni *et al.* [3-5] described the effects of locoregional injections of very low doses of IL-2 in a CE-2 sarcoma in syngeneic BALB/c mice. In the presence of otherwise non-reacting lymphocytes from tumor-bearing mice, immunorecognition of tumor cells was enhanced and specific and nonspecific host effector mechanisms were recruited. This resulted in almost complete tumor-growth inhibition and the development of a specific immunologic memory. Higher doses of IL-2 did not improve the effect.

Based on these results, a clinical study was started by Cortesina *et al.* [6] in patients with recurrent head and neck squamous-cell carcinoma (HNSCC). In ten patients, treated with repeated perilymphatic injections of 200 U of Jurkat-IL-2, three complete responses (duration of 4-6 months) and three partial responses were recorded. No tumor regression was seen in the two patients who had prior bilateral lymph-node dissection.

Local IL-2 immunotherapy appears to be highly suited for the treatment of locally advanced HNSCC with its superficial location (making it accessible to injection) and juxtaposition to an extensive lymph-node network (and thus a pool of lymphocytes). With current surgical, radiotherapeutic, and chemotherapeutic treatment methods, the prognosis for these patients is poor [7-12]. Besides this, in HNSCC patients, immune reactivity is frequently depressed [13,14]. IL-2 might help to overcome this. The addition of IL-2 to *in vitro* cultures restores the diminished lymphocyte function and counteracts immune suppressive effects from the HNSCC patient's serum [15].

The present study was designed to verify the value of treatment with a low dose of recombinant IL-2 injected perilymphatically in HNSCC, as described by Cortesina *et al.* [6]. Contrary to them, however, we selected a nonpretreated patient group, because of the expected relevance of an intact lymph-node system (*i.e.*, not damaged by prior therapy). Tumor biopsies for histopathological and immunohistochemical studies were taken before and after IL-2 treatment to obtain additional diagnostic, prognostic, and biological information.

Materials and methods

Patients

Fifteen patients with histologically proven, locally far-advanced, nonpretreated HNSCC, not amenable to primary therapy with curative intent, gave their informed consent and entered into this study between July 1988 and January 1989. Eligible patients met the following requirements: a Karnofsky performance status of 70% or more, an expected survival of at least 3 months, normal hematologic parameters, adequate liver, kidney and cardiac function, no other significant medical conditions requiring ongoing therapy, no organ allografts, no distant metastases, and no second malignancies. Patient characteristics are summarized in Table 1.

Table 1. *Patient characteristics and treatment with perilymphatic injections of IL-2 in 15 patients with locally far-advanced HNSCC.*

		Tumor			IL-2 injections			
Pat. no.	Age, sex	Location	TNM stage[a]	Performance status[b]	Site[c]	Dose/ site[d]	Courses	Response[e] (day 28)
1	57,F	Oropharynx	T3N2	80%	bi	0.2	1	SD
2	48,M	Floor of mouth	T4N3	100%	uni	0.2	2	SD
3	67,M	Floor of mouth	T4N3	70%	uni	0.2	1	PD
4	55,M	Hypopharynx	T4N3	100%	uni	0.2	2	SD
5	58,M	Hypopharynx	T4N3	90%	bi	0.2	1	SD
6	74,F	Floor of mouth	T4N1	80%	uni	0.2	1	SD
7	61,M	Hypopharynx	T4N3	70%	uni	0.2	1(8 days)	-
8	78,M	Oropharynx	T4N3	100%	bi	0.2	1(9 days)	-
9	51,M	Floor of mouth	T4N3	90%	bi	0.4	1	PD
10	43,M	Oropharynx	T4N1	90%	uni	0.4	1	PD
11	67,M	Oropharynx	T4N3	80%	bi	0.4	1	SD
12	65,M	Oropharynx	T4N0	90%	uni	0.4	1	SD
13	73,F	Oropharynx	T2N3	100%	bi	0.4	1	SD
14	50,M	Oral cavity	T4N3	100%	bi	0.4	1	PD
15	60,F	Oropharynx	T4N2	90%	uni	0.4	1	SD

[a] Indicates tumor size at the start of IL-2 treatment. All were M0.

[b] Karnofsky score.

[c] uni, unilateral; bi, bilateral.

[d] Dose in μg of IL-2. In cases of bilateral injections, twice this dose was administered.

[e] SD, stable disease; PD, progressive disease.

Treatment

Recombinant IL-2 was provided by Glaxo IMB (Geneva, Switzerland). The specific activity was 10^6-10^7 U/mg protein. IL-2 was diluted in saline, containing 10% human serum albumin. Perilymphatical bolus injections were given on 10 consecutive days ipsilateral to the side with the tumor, or bilateral in cases of midline or bilateral disease. The injection site (1.5 cm from the insertion of the sternocleidomastoid muscle to the mastoid tip at a depth of 1.5 cm) is based on lymphographic studies, as mentioned by Cortesina *et al.* [6]. The first eight patients received IL-2 0.2 µg/site/day, and, as no response was seen in this group, the seven subsequent patients received IL-2 0.4 µg/site/day. Patients who received bilateral injections got twice this dose. This course was scheduled to be repeated after 30 days in responding patients. The tumor response was evaluated on days 10 and 28 with physical examination and/or CT-scan, and determined according to WHO criteria.

Tissue specimen

Surgical biopsies from the primary tumor both prior to the start of treatment and on day 18 were obtained from 10 patients. A fresh specimen from most of these biopsies was deep frozen and stored in liquid nitrogen until use.

The histopathological analyses were centrally performed on paraffin sections, and included documentation of the pathological diagnosis and grading according to the modified Jakobsson system for squamous-cell carcinoma. In this semi-quantitative grading scheme, six histological parameters of the tumor cell population and the tumor-host interface are scored on a 1 to 4 point scale [16]. The cellular infiltrate in the tumor-surrounding tissue was examined for overall intensity of mononuclear cells and the intensity of particular subsets of cells (lymphocytes, plasma cells, neutrophils, eosinophils, macrophages). The intensity was scored as - (no or only very few cells), + (occasional patch), ++ (patchy rim), or +++ (continuous rim of infiltrating cells).

Immunohistochemical analysis

The expression of human leukocyte antigens (HLA) by tumor cells was determined using an indirect immunoperoxidase procedure with 4 µm thick cryostat sections. If these were not available, paraffin sections were used. The peroxidase label was visualized by 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Sections were counterstained with Harris's hematoxylin. The intensity of the staining was scored from negative (-) to highly positive (+++). Expression of HLA antigens by tumor cells was estimated as a percentage of the total number of tumor cells, and scored in one of the following

categories: 0-5%, 6-25%, 26-50%, 51-75%, and 76-100%. Phenotyping of the lymphocytic infiltrate was performed according to standard procedures on frozen sections. The slides were read by two different observers; agreement was above 90%.

Antibodies

The following panel was used for evaluating HLA antigen expression: the monoclonal antibody (MAb) W6/32, recognizing all ß2-microglobulin-bound HLA class I antigens [17]; a MAb against ß2-microglobulin (clone B.11.G6, Dr. B. Malissen, Marseille, France); HCA2, a MAb recognizing mainly heavy (H) chains of class I HLA-A loci [18]; HC10, a MAb recognizing mainly H chains of class I HLA-B and C loci [18]; RaHC, anti-H chain rabbit serum recognizing HLA-A, B, and C H chains [18]; and three rabbit sera recognizing HLA class II antigens, raised against HLA-DR, -DP, and -DQ, respectively (all provided by Dr. H.L. Ploegh, Amsterdam, The Netherlands). Except for W6/32 and anti-ß2-microglobulin, all antibodies are applicable to both cryostat and paraffin sections. In addition T11 (anti-CD2), Leu4 (anti-CD3), Leu3 (anti-CD4), WT82 (anti-CD8), B1 (anti-CD20), Leu7 [natural killer (NK) cells], and anti-HLA-DR were used for phenotyping the infiltrate [19].

Results

Clinical evaluation

Thirteen patients completed the 10-day course. In patient 7, treatment was stopped after 8 days because of progressive dyspnea due to tumor progression. Patient 8 was withdrawn from the study after 9 days because of the development of a double-sided pneumonia and an arterial embolism in the right femoral artery. This patient died of pulmonary failure 4 weeks after starting IL-2 treatment. In patient 13, an increasing swelling and redness of the neck lymph-node mass during treatment resulted in a rupture with loss of necrotic material on day 11. There were no signs of infection.

Thirteen patients were evaluable on day 28. None of them showed signs of tumor regression. Stable disease was determined in nine patients and progressive disease in four patients. Patients 2 and 4 had subjective improvement of symptoms. Both received a second treatment course, without inducing an objective response.

The treatment was well tolerated. No side effects were recorded.

Subsequently, 11 patients received chemotherapy and/or radiotherapy. Patient 4 had a complete response after combined chemo- and radiotherapy, and was still free of tumor 17

months after the IL-2 treatment. For all other patients, the median survival from the start of the IL-2 treatment was 7 months.

Histopathological evaluation

From ten patients (nos. 1,2,3,4,5,9,10,11,14,15), tumor biopsies from both before and after IL-2 treatment were available (Table 2). All had moderately differentiated squamous-cell carcinoma. The mean of the Jakobsson score before treatment was 13, ranging from 11 to 14, except in patient 1 who had a score of 20 points.

A patchy rim of mononuclear cells (++) was observed in the tumor-surrounding tissue in seven out of ten pretreatment biopsies. No significant changes in the intensity of the mononuclear cell infiltrate were found following IL-2 treatment (Table 2). The composition of the mononuclear cell infiltrate also did not change significantly after IL-2 treatment. Lymphocytes were the most frequently present cells, followed by plasma cells. Eosinophils were sparse, and their number did not increase after IL-2 treatment (data not shown).

Additional immunohistochemical phenotyping of the lymphocytic infiltrate in two representative patients (nos. 9 and 11) showed the following profile. The large majority of the lymphocytes were T cells; the CD4/CD8 ratio was about 1:1; approximately 75% of the T cells were HLA-DR positive; B cells (B1) and NK cells (Leu7) were sporadically observed. No differences between treated and nontreated lesions were found (data not shown).

Expression of HLA class I and II antigens by tumor cells

The levels of expression obtained using the different antibodies for HLA class I antigens agreed. W6/32 and HC10 gave the most intense staining in cryostat and paraffin sections, respectively. The results for HC10 are presented in Table 2. In six of ten tumor biopsies, more than 50% of the tumor cells showed HLA class I antigen expression. Only one sample (no. 1), which also had a very high Jakobsson score, was negative for HLA class I antigens (0-5%). No selective loss of HLA-B was seen. After IL-2 treatment, no significant changes in HLA class I expression could be detected.

Equal levels of expression were obtained with the antibodies against HLA-DR, -DP, and -DQ. The results for anti-HLA-DR are presented in Table 2. Eight of ten samples were HLA class II-negative (0-5%), both before and after IL-2 treatment. Only two biopsies (nos. 9 and 10) had locally positive tumor cells before and after IL-2 treatment. These tumors both had a relatively low Jakobsson score. There was no striking intensity of the mononuclear cell infiltrate in the HLA class II-positive tumors.

Table 2. *Histopathological evaluation and HLA antigen expression on pre- and post-treatment tumor biopsies from 10 patients with HNSCC, treated with perilymphatic injections of IL-2.*

Pat. no.[a]	Histopathological features			Intensity of mononuclear cell infiltrate[d]		HLA antigen expression by tumor cells[e]			
	Grade[b]	Jak score[c]				class I		class II	
		Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
1	Moderately	20	18	+	+	0-5%	0-5%	0-5%	0-5%
2	Moderately	13	12	+	++	51-75%	51-75%	0-5%	0-5%
3	Moderately	13	12	++	+	6-25%	26-50%	0-5%	0-5%
4	Moderately	13	14	++	+++	26-50%	6-25%	0-5%	0-5%
5	Moderately	12	15	++	++	76-100%	76-100%	0-5%	0-5%
9	Moderately	11	10	++	+	76-100%	76-100%	26-50%	26-50%
10	Moderately	11	17	+++	n.i.	76-100%	76-100%	51-75%	6-25%
11	Moderately	14	17	++	+++	76-100%	76-100%	0-5%	0-5%
14	Moderately	14	11	++	n.i.	6-25%	51-75%	0-5%	0-5%
15	Moderately	11	8	++	++	76-100%	76-100%	0-5%	0-5%

Pre-, pretreatment; post-, post-IL-2 treatment on day 18.

[a] Numbers corresponding to Table 1.

[b] Grade of squamous-cell carcinoma, pretreatment; moderately differentiated.

[c] Jakobsson score.

[d] –, no or only very few mononuclear cells; +, occasional patch; ++, patchy rim; +++, continuous rim of infiltrating cells; n.i., not interpretable due to absence of tissue surrounding the tumor in the specimen.

[e] Percentage of positive cells; results from MAb HC10 (HLA class I), and anti-HLA-DR serum (HLA class II).

Discussion

In 15 patients with nonpretreated locally advanced HNSCC, we did not observe tumor shrinkage after a 10-day treatment course with low doses of IL-2 injected perilymphatically. The observation period used to detect a response to IL-2 treatment was 4 weeks, which is short. In prior studies on this treatment modality [6,20], however, clinical responses and histological changes occurred within 4 weeks after the start of treatment. Our findings are in contrast with Cortesina's results [6] with perilymphatic

injections of Jurkat-IL-2 in recurrent inoperable HNSCC, which might be partly explained by a difference in patient characteristics. The current study concerned primary disease and the size of the tumor masses, as indicated by the TNM stage, was considerably larger than in Cortesina's patients.

Our histopathological findings corresponded to the clinical results, since no changes were found after treatment. Furthermore, in the post-IL-2 biopsies, we did not find an increase in the mononuclear cell infiltrate, an eosinophilic infiltration, or necrosis of tumor cells, as described in responding patients in other series [20,21].

The low level of HLA class II antigen expression in HNSCC has been described before by Esteban *et al.* [22], and might be disadvantageous with regard to tumor control. HLA class II antigens are essential for antigen presentation to CD4-positive T cells, and a correlation between expression of HLA-DR antigens by tumor cells and a favorable response to IL-2-based immunotherapy has been suggested [21,23]. In HNSCC Esteban *et al.* [22] found a correlation between HLA class II antigen expression and a well-differentiated pattern and also a better prognosis. The low frequency of expression by HNSCC lesions, however, limits the utility of HLA class II as a marker to predict the prognosis or the effect of immunotherapy.

HLA class I antigen expression, on the other hand, was found in nine of the ten cases in the current study. This might suggest susceptibility to killing by CD8-positive cytotoxic lymphocytes, one of the supposed mechanisms of immunotherapy. Besides, a low expression of HLA class I antigens is associated with a poor prognosis in melanoma [24], and with a poor differentiation in HNSCC [22]. Despite the high HLA class I expression in our study, however, the prognosis was poor.

Further studies are required to establish the true value of locoregional treatment with low doses of IL-2 in HNSCC. Our findings illustrate the feasibility of repeated representative histological tumor analysis in this kind of cancer, which might provide more relevant information about the mechanisms of action of immunotherapy and factors predictive of prognosis.

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Chapter 5

Recombinant interleukin-2 and PEG-interleukin-2 in locoregional immunotherapy of established guinea-pig line-10 tumors

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Mattijssen V, De Mulder PHM, Balemans LTM, Steerenberg PA. Efficacy of polyethylene glycol-modified interleukin-2 in locoregional immunotherapy of guinea-pig tumors. *Proc AACR* 1992; 33:329 (abstract).

Summary

Background: Polyethylene glycol-modified interleukin-2 (PEG-IL-2) represents a cytokine with prolonged circulatory half-life and increased antitumor activity as compared to unmodified IL-2 after systemic administration. We studied whether PEG-IL-2 would also be advantageous in locoregional immunotherapy of established tumors.

Methods: The syngeneic guinea-pig line-10 tumor model was used, characterized by progressive local tumor growth following intradermal inoculation, and regional lymph-node metastases within 7 days. Intratumoral and/or perilymphatic injections with IL-2 or PEG-IL-2 were started on day 7 after inoculation, in animals with palpable tumors. Treatment groups consisted of 5-6 animals.

Results: Combined intratumoral and perilymphatic injections with PEG-IL-2 caused significant growth inhibition of both the primary tumor and the regional lymph-node metastases at lower doses and with less frequent administration than IL-2. The best schedule for PEG-IL-2 included 3 injections a week for 5 weeks, resulting in cure of 4/17 and 5/5 ($p<0.01$) animals at the 2 most efficient dose levels tested (60,000 and 200,000 U, respectively). Subsequent experiments indicated that the intratumoral and not the perilymphatic injection route was essential for the obtained antitumor effect. Moreover, 12/12 animals cured after PEG-IL-2 treatment rejected a rechallenge with line-10 tumor cells. No cures were seen after IL-2 treatment.

Conclusion: Intratumoral PEG-IL-2 injections can cure guinea-pigs with palpable experimental tumors and regional lymph-node micrometastases, and induce protective systemic antitumor activity, without signs of toxicity.

Introduction

Interleukin-2 (IL-2), a cytokine produced by activated T cells, is significant for the growth and function of a variety of immune cells [1-5]. Endogenous IL-2 mediates its effect locally at the cell-cell interaction level, and its activity is not normally detectable in human serum. Since recombinant human IL-2 has become available, its potential as an immune stimulant in anticancer immunotherapy has been studied on a large scale. Most studies concern intravenous (i.v.), *i.e.* systemic, administration of IL-2 either alone or in combination with other cytokines, or adoptive cellular therapy. The very short circulatory half-life, being 13 min initially after i.v. bolus injection followed by a slower phase with a half-life of 85 min, is well documented [6]. Using high doses and long-term i.v. administration, antitumor effects are obtained with IL-2-based therapy especially in metastatic malignant melanoma and renal-cell carcinoma [7]. However, the number of responding patients in this and other trials is limited and the treatment is accompanied by serious toxicity. Although subcutaneous (s.c.) administration, resulting in lower but fairly constant serum IL-2 levels for about 8 h [6], may reduce toxicity [8], a widely applicable regimen for treating cancer patients has not yet been realized. Furthermore, the mechanisms by which IL-2 mediates antitumor effects *in vivo*, *i.e.* activation of a circulating cell population such as lymphokine-activated killer cells, or stimulation of immune reactions directly at the tumor site, are largely unknown. Meanwhile it is questionable whether high-dose i.v. or s.c. IL-2 administration is always the most beneficial approach. Alternative administration routes are being explored, such as IL-2 injections intralymphatically on the dorsum of the foot [9] or into the splenic or hepatic artery [10] in an attempt to enhance immunostimulation *in vivo*. On the other hand, locoregional IL-2 application should be considered in certain malignancies with local tumor spread. This might, in contrast to systemic administration, promote a more physiological immune activation, providing a higher IL-2 concentration at the tumor site to stimulate infiltrating immune cells, and avoiding systemic toxicity [11]. The efficacy of repeated injections with fairly low doses of natural IL-2 or recombinant IL-2 into or next to the tumor lesion, or around the tumor-draining lymph nodes, has been described in several syngeneic animal tumor models [12-14], in spontaneous bovine ocular carcinoma [15], in human bladder cancer [16], and head and neck squamous-cell carcinoma [17]. In addition, intracavitary application was studied in patients with malignancies limited to the peritoneal cavity [18].

Recently, chemical modification of IL-2 with the water-soluble polymer polyethylene glycol resulted in a substance with enhanced solubility and a 10- to 20-fold prolonged circulatory half-life [19,20]. The *in vitro* specific activity of PEG-IL-2 is qualitatively

unchanged, but quantitatively 2- to 3-fold lower than that of unmodified IL-2. However, this does not correlate with its *in vivo* activity, which may be related to the duration of exposure. In 3 murine tumors i.v. administered PEG-IL-2 showed a significantly higher antitumor activity than IL-2, and a less frequent administration schedule proved to be sufficient [21]. So far, no data are available on locoregional PEG-IL-2 application in animal studies or patients.

We have investigated in a syngeneic guinea-pig tumor model whether PEG-IL-2 would be particularly advantageous in locoregional immunotherapy as well. The objectives of this study were: 1. to compare the antitumor activity of IL-2 and PEG-IL-2 when injected intratumorally and perilymphatically in established tumors; 2. to determine the optimal administration route and schedule for locoregional PEG-IL-2 therapy; and 3. to observe the development of systemic antitumor activity after locoregional PEG-IL-2 application.

Materials and methods

Animals

Female Sewall-Wright inbred strain-2 guinea-pigs were obtained from the National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD. The animals were caged in groups of 6 or less and fed guinea-pig rations and water *ad libitum*. They were used when 2 to 3 months old, weighing 400 to 600 g.

Tumor

Line-10 tumor cells, syngeneic to strain-2 guinea-pigs, were kindly provided by Dr. H.J. Rapp and Dr. B. Zbar (National Cancer Institute, Bethesda, MD). This hepatocellular carcinoma was, as one in a series of tumors, induced by oral administration of diethylnitrosamine [22], and converted to the ascites form. Intradermal inoculation of 1×10^6 tumor cells in the anterior flank led to progressive local tumor growth, development of regional axillary lymph-node metastases within 1 week, and lung metastases resulting in death after 60-90 days [23]. The tumor was weakly immunogenic. The tumor-cell line was maintained as frozen stock, and propagated intraperitoneally. Tumor cells were used in their 12th passage.

Therapeutics

Highly purified recombinant IL-2 (Proleukin) and PEG-IL-2 were provided by Eurocetus, Amsterdam, The Netherlands. The PEG-IL-2 used has a degree of modification equivalent to 2-3 PEG M_r 7,000 molecules/IL-2 molecule. The apparent average molecular weight of this species was M_r 160,000 by size-exclusion chromatography [19]. The specific activities of the IL-2 and PEG-IL-2 were 18.0×10^6 and 9.4×10^6 U/mg IL-2 protein, respectively. Stock concentrates of 1×10^6 U/ml were prepared from lyophilized IL-2 and PEG-IL-2 by reconstitution in sterile water and dilution in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA), and stored at -20°C. Before use IL-2 and PEG-IL-2 were further diluted in PBS containing 0.1% (w/v) BSA.

Treatment schedules

Guinea-pigs were injected intradermally in the left anterior flank with 1×10^6 line-10 tumor cells. After 7 days the animals, bearing established tumors, were randomly divided into treatment groups of 5-6 animals, and 0.2-ml injections with IL-2 or PEG-IL-2 were started. In the first series of experiments both intratumoral and perilymphatic injections were given at different schedules and dose levels. The perilymphatic injection was administered between the tumor inoculation site and the tumor-draining axillary lymph nodes. In subsequent experiments the influence of the injection site was evaluated by treating animals either intratumorally or perilymphatically. Control animals received injections with PBS containing 0.1% (w/v) BSA in a similar way, or no injections at all. To evaluate the effect of PEG on tumor growth, one separate group received injections with PEG M_r 6,000 (Merck, Darmstadt, Germany) in PBS without IL-2. The tumors on the flank were measured weekly from day 7 with calipers in 3 directions and the volume was calculated. The incidence of palpable axillary lymph-node metastases was recorded. The animals were observed until the diameter of the axillary lymph-node mass exceeded 25 mm, at which time they were killed.

Treated animals without signs of tumor growth on day 49 received a second challenge with 1×10^6 line-10 tumor cells intradermally in the contralateral (right) flank to test the development of systemic antitumor activity. As a control, untreated animals were inoculated at the same time. Tumor growth was measured weekly.

Treated animals were considered to be cured if tumor-free on day 84 after tumor-cell inoculation.

Statistical analysis

Students' *t*-test was used to determine significant differences in tumor growth between treatment and control groups of animals. In case of insufficient homogeneity of variances, the Welch correction with respect to the degrees of freedom was applied. Differences in survival between control and treatment groups were evaluated by Fisher's exact test.

Results

Daily intratumoral and perilymphatic administration of IL-2 or PEG-IL-2

The activity of IL-2 and PEG-IL-2 in locoregional immunotherapy was compared first in a daily administration schedule. A combined intratumoral and perilymphatic approach was chosen, because in the model used microscopic axillary lymph-node metastases have already developed before day 7 [23].

On day 7 after line-10 tumor inoculation, animals had established tumors on the flank with a diameter of 6-8 mm. In control animals the tumor always showed progressive growth with disruption of the skin and ulceration, while axillary lymph-node metastases became palpable on day 21 to 28 and reached a diameter of approximately 25 mm on day 42. No differences in tumor growth were observed between nontreated animals and those receiving PBS+BSA control injections (Fig. 1). The observation period in the Figures up to day 42 marks the point at which the control animals had to be killed because of extensive regional lymph-node metastases.

Figure 1 shows the results of an experiment comparing the effects of 8 daily IL-2 and PEG-IL-2 injections, with doses ranging from 3,000 to 60,000 U per injection site, on the intradermal growth of line-10. Treatment with IL-2 resulted in a significant inhibition of tumor growth at the dose level of 60,000 U (Fig. 1a, $p<0.01$). With PEG-IL-2, however, a similar inhibition was achieved at a dose level of 9,000 U (Fig.1b, $p<0.01$). This was confirmed in a similar experiment (tumor-growth curves not shown).

Table 1 summarizes the data on lymph-node metastases of all experiments performed. When daily treatment schedules were used, the incidence of palpable axillary lymph nodes was delayed by 14 days or more in 4/26 (15%) of the IL-2-treated animals and in 33/50 (66%) of the animals injected with PEG-IL-2 (Table 1, treatment groups 1-4 and 7-10, respectively). Only 1 animal, treated with 60,000 U PEG-IL-2, was cured (Table 1, group 10).

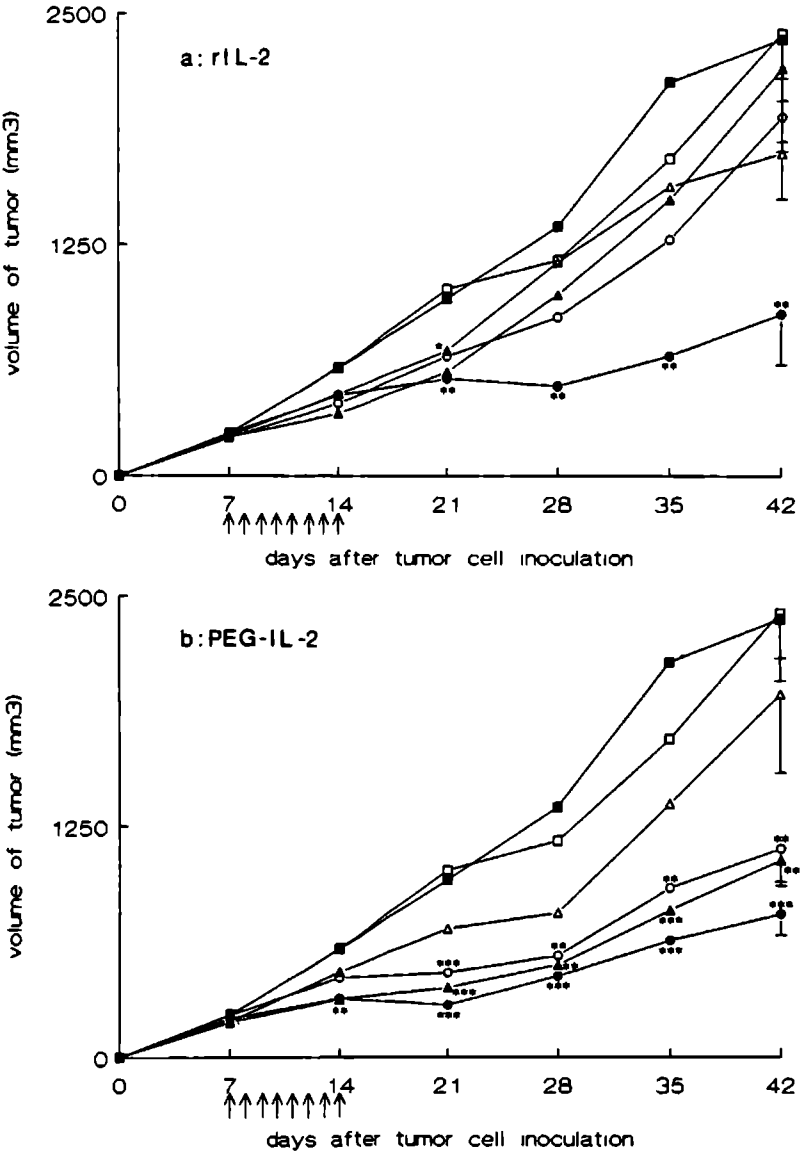

Figure 1. Growth of intradermal line-10 tumors following daily (day 7-14) intratumoral and perilymphatic injections with IL-2 (*a*) or PEG-IL-2 (*b*). The doses indicated are per injection site, per day. ■, Control PBS+BSA; □, control untreated; Δ, 3,000 U; ▲, 9,000 U; O, 30,000 U; ●, 60,000 U. Arrows, treatment days. Points, mean values of 5 animals; bars, SEM. Statistically significant differences compared to the untreated group are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table 1. *Metastatic growth and survival after locoregional IL-2 or PEG-IL-2 treatment of established intradermal line-10 tumors in guinea-pigs*[a].

	Treatment[b]		Development of palpable axillary lymph-node metastases; delay in days[c]				Tumor-free/ number treated (%) on day 84
	Dose and schedule	Site	0	7	14	≥21	
	IL-2						
1.	3,000 U, daily, day 7-14	i.t.+p.l.	3/5	2/5			0/5
2.	9,000 U, daily, day 7-14	i.t.+p.l.		4/5	1/5		0/5
3.	30,000 U, daily, day 7-14	i.t.+p.l.	1/5	4/5			0/5
4.	60,000 U, daily, day 7-14	i.t.+p.l.	1/11	7/11	1/11	2/11	0/11
5.	60,000 U, 2×/wk, for 5 wks	i.t.+p.l.	5/6		1/6		0/6
6.	60,000 U, 3×/wk, for 5 wks	i.t.+p.l.	6/6				0/6
	PEG-IL-2						
7.	3,000 U, daily, day 7-14	i.t.+p.l.	2/11	8/11	1/11		0/11
8.	9,000 U, daily, day 7-14	i.t.+p.l.		3/11	5/11	3/11	0/11
9.	30,000 U, daily, day 7-14	i.t.+p.l.			9/11	2/11	0/11
10.	60,000 U, daily, day 7-14	i.t.+p.l.	1/17	3/17	9/17	4/17	1/17 (6)
11.	60,000 U, 2×/wk, for 5 wks	i.t.+p.l.	1/6	2/6	3/6		0/6
12.	60,000 U, 3×/wk, for 5 wks	i.t.+p.l.			2/17	15/17	4/17 (24)
13.	200,000 U, 3×/wk, for 5 wks	i.t.+p.l.				5/5	5/5 (100)[d]
14.	60,000 U, 3×/wk, for 5 wks	i.t.	1/6	1/6		4/6	2/6 (33)
15.	60,000 U, 3×/wk, for 5 wks	p.l.	6/6				0/6
16.	60,000 U, 3×/wk, for 5 wks	p.l.[e]		3/6	2/6	1/6	0/6

[a] Summary of 5 separate experiments.

[b] Treatment with intratumoral (i.t.) and/or perilymphatic (p.l.) IL-2 or PEG-IL-2 injections was always started on day 7 after intradermal inoculation with 1×10^6 line-10 tumor cells in the flank. Dose indicated in U per injection day, and per injection site. Treatment regimens 4,7,8,9,10 and 12 were tested in more than one experiment.

[c] Delay as compared to the control group of animals in the individual experiment, indicated as number/total treated. Control groups of animals were scored positive when ≥80% of animals had palpable axillary lymph nodes (≥6 mm). In the separate experiments this was on day 21, 28, or 35.

[d] Significant difference as compared to control group ($p<0.01$).

[e] After excision of the tumor on the flank on day 7.

Intermittent intratumoral and perilymphatic administration of IL-2 or PEG-IL-2

To test the schedule dependency, in another experiment the daily treatment course was compared to schedules with less frequent, but more prolonged, administration. Injections were given intratumorally and perilymphatically at a dose level of 60,000 U per injection site per day. PEG-IL-2 injected twice weekly (tumor-growth curve not shown) or 3 times weekly for 5 weeks (Fig. 2) had a significant tumor-growth-inhibiting effect ($p<0.05$ and $p<0.01$ respectively), while IL-2 using these schedules had no significant effect on the intradermal tumor (Fig. 2), nor on the lymph-node metastases (Table 1, treatment group 5-6). Treatment with 3 PEG-IL-2 injections per week was equal to 8 daily 60,000 U PEG-IL-2 injections with regard to inhibition of the intradermal tumor growth (curve of daily treatment not shown in Fig. 2). Summarizing the results of the different experiments, however, the former schedule gave a more pronounced delay in development of palpable lymph nodes (Table 1, group 12 and 10 respectively). With the 3-times-weekly schedule a delay of at least 21 days was observed in 15/17 (88%) animals. Moreover, 4/17 (24%) animals in group 12 were cured.

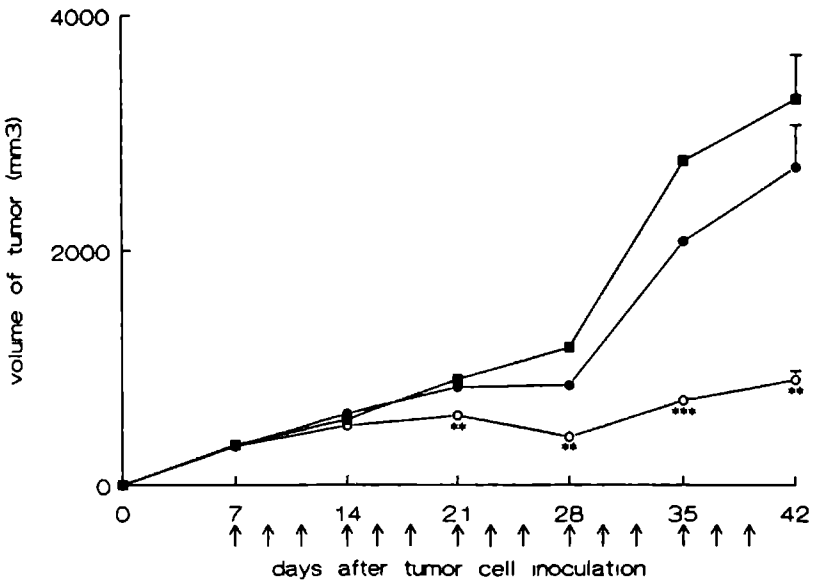

Figure 2. Growth of intradermal line-10 tumors following 3-times-weekly treatment with IL-2 or PEG-IL-2. Intratumoral and perilymphatic injections with IL-2 (●) or PEG-IL-2 (○) were given 3 times weekly for 5 weeks (arrows) at a dose of 60,000 U per injection site, per day. ■, Control, untreated; points, mean values of 6 animals; bars, SEM. Statistically significant differences compared to the control group are indicated: **, $p<0.01$; ***, $p<0.001$.

Relevance of injection site

The relevance of the injection site was evaluated by treating animals either intratumorally or perilymphatically or at both locations with the optimal schedule so far, *i.e.* PEG-IL-2 injections (60,000 U) 3 times weekly. In addition, in one group only perilymphatic injections were given after the intradermal tumor was excised under general anaesthesia on day 7. Intratumoral injections alone, like combined intratumoral and perilymphatic injections, suppressed intradermal tumor growth (Fig. 3). Furthermore, the development of lymph-node metastases was delayed for at least 21 days in 4/6 (67%) animals, and 2/6 (33%) animals were cured (Table 1, group 14). Perilymphatic treatment, on the other hand, had no effect at all on the intradermal tumor on the flank (Fig. 3), and did not suppress the lymph-node metastases (Table 1, group 15). Even after excision of the primary tumor on day 7, the perilymphatic injections resulted in a variable, but mostly (5/6 animals) only 7- to 14- day delay in the growth of the axillary lymph-node metastases (Table 1, group 16).

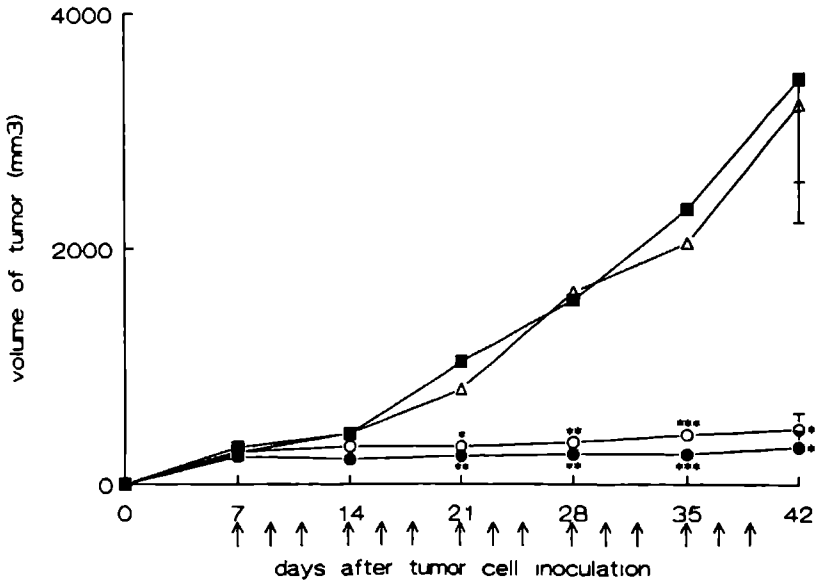

Figure 3. Growth of intradermal line-10 tumors following treatment with PEG-IL-2 at different injection sites. PEG-IL-2 injections were given 3 times weekly for 5 weeks (arrows) at a dose of 60,000 U per injection site per day. ■, Control, PBS+BSA; ○, intratumoral and perilymphatic; ●, intratumoral; Δ, perilymphatic. Points, mean values of 6 animals; bars, SEM. Statistically significant differences compared to the control group are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Optimizing the PEG-IL-2 dose

So far, the results obtained with doses ranging from 3,000 to 60,000 U showed a dose-response relation for locoregional PEG-IL-2 treatment, and application 3 times weekly proved to be the best schedule. To further improve the efficacy the PEG-IL-2 dose was escalated to 200,000 U. The results of this experiment are shown in Figure 4. In 5/5 animals no tumor was palpable on day 28, and no lymph-node metastases developed (Table 1, group 13). All 5 animals were cured ($p<0.01$).

In all experiments in this study, including the highest dose level of 200,000 U PEG-IL-2, therapy was well tolerated by the guinea-pigs. No adverse effects were seen at the injection site, and no premature deaths occurred.

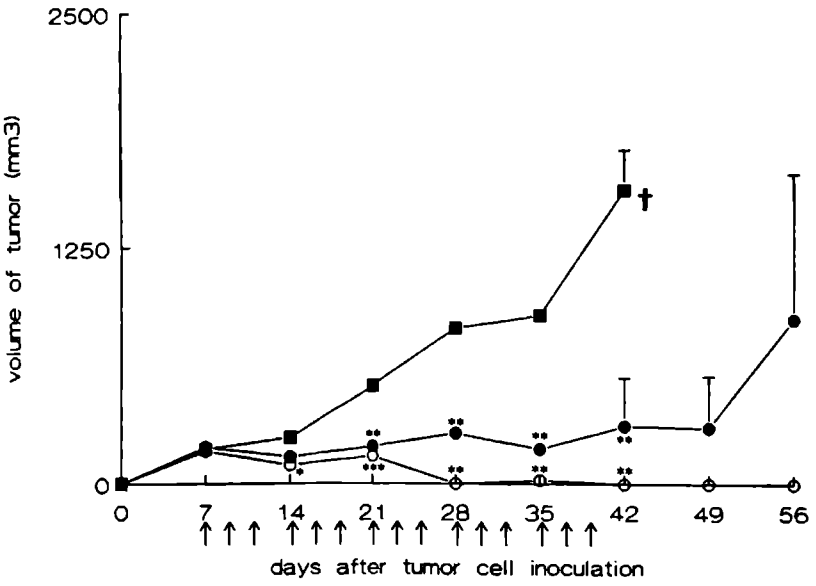

Figure 4. Growth of intradermal line-10 tumors following treatment with PEG-IL-2 at different dose levels. Intratumoral and perilymphatic PEG-IL-2 injections were given 3 times weekly for 5 weeks (arrows) at a dose of 60,000 U (●) or 200,000 U (○) per injection site per day. ■, Control, PBS+BSA. Points, mean values of 5 animals; bars, SEM. †, death. Statistically significant differences compared to the control group are indicated: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Rechallenge with line-10 tumor cells

In total, 16 PEG-IL-2-treated animals (5 treated daily and 11 treated 3 times weekly; Table 2) showed no signs of tumor growth on day 49 in the individual experiments, and were given a second challenge with 1×10^6 viable line-10 tumor cells in the contralateral (right) flank. Three out of the 5 animals undergoing daily treatment (groups 8 and 10) rejected the rechallenge, however 2 of these 3 ultimately showed tumor growth at the primary inoculation side, and only 1 proved to be cured. All 11 animals receiving a rechallenge after PEG-IL-2 treatment 3 times weekly (group 12-14) were resistant to the line-10 tumor, and remained tumor-free. The nonpretreated control animals challenged at the same time all showed progressive tumor growth. None of the IL-2-treated animals was eligible for a second tumor challenge on day 49, because all had palpable lymph-node metastases.

Table 2. *Second challenge with 1×10^6 line-10 tumor cells in guinea-pigs following locoregional PEG-IL-2 treatment.*

	PEG-IL-2 treatment		Number resistent[a]/ number challenged	Tumor-free[b] on day 84/number challenged
	Dose and schedule	Site		
8[c]	9,000 U, daily, day 7-14	i.t.+p.l.	1/3	0/3
10	60,000 U, daily, day 7-14	i.t.+p.l.	2/2	1/2
12	60,000 U, 3x/wk, for 5 wks	i.t.+p.l.	4/4	4/4
13	200,000 U, 3x/wk, for 5 wks	i.t.+p.l.	5/5	5/5
14	60,000 U, 3x/wk, for 5 wks	i.t.	2/2	2/2

Animals without signs of tumor growth or lymph-node metastases after locoregional PEG-IL-2 treatment were rechallenged in the contralateral (right) flank on dag 49 after tumor inoculation.

[a] Resistent to second tumor challenge in right flank.

[b] Tumor-free on original inoculation (left) side on day 84 after this inoculation.

[c] Numbers corresponding to Table 1.

Discussion

In the present study PEG-IL-2 proved to have clear advantages in locoregional anticancer immunotherapy as compared to unmodified IL-2. PEG-IL-2 showed significant tumor-growth-inhibiting effects, regarding both the primary tumor and the regional lymph-node metastases, at lower doses and with less frequent administration than IL-2. The best results

were obtained with PEG-IL-2 injected 3 times weekly, while IL-2 showed a tumor-growth-inhibiting effect only if administered daily. This finding is in concordance with optimal treatment schedules for i.v. IL-2 or PEG-IL-2 administration [21]. Schedules with less frequent administration provide practical advantages in patient treatment. More important, however, is the fact that locoregional PEG-IL-2 treatment resulted in cure of guinea-pigs with established tumors. Furthermore, cured animals rejected rechallenges with viable tumor cells, which suggests the development of systemic immunity. From the schedules tested, the best results (5/5 cured) were obtained with 200,000 U PEG-IL-2 injected both intratumorally and perilymphatically 3 times weekly for 5 weeks. The total dose received by these animals was 4×10^5 U/day, or 12×10^5 U/wk. This is about 8×10^5 U/kg/day, or 24×10^5 U/kg/wk, which was tolerated by the guinea-pigs without any sign of toxicity.

The tumor model used in this study is a suitable experimental model for locoregional immunotherapy for several reasons. The syngeneic guinea-pigs are immunologically competent hosts. Line-10 is an undifferentiated carcinoma, characterized by progressive local tumor growth at the inoculation site, and fast development of regional lymph-node metastases. In this respect it resembles human head and neck squamous-cell carcinoma, which is a good candidate for locoregional IL-2 immunotherapy [17]. To obtain a reasonable reflection of clinical practice, treatment protocols in this study were always started in animals with established tumors on day 7, when microscopic axillary lymph-node metastases have already developed [23]. This was actually confirmed in the experiment with surgical removal of the intradermal tumor on day 7. In spite of perilymphatic treatment, large axillary lymph-node metastases developed in 5/6 animals. Whereas Vaage [14] found a relation between responsiveness to local IL-2 immunotherapy and strong immunogenicity in mouse mammary tumors, the line-10 tumor is only weakly antigenic. Following a challenge with irradiated tumor cells, a very weak DTH responsiveness is observed [24]. Spontaneous regression, or rejection of a second challenge after surgical resection of a 1-cm primary tumor, is never observed.

The increased antitumor activity *in vivo* of locally administered PEG-IL-2 as compared to IL-2 can probably be explained by a prolonged exposure time. Therapeutic effects of sustained peritumoral IL-2 release were previously described by Bubeník *et al.* [25], using IL-2-producing lymphoid cells transformed by IL-2 cDNA, and by Vaage and Mayhew [26], using IL-2 liposome-gel preparations. PEG itself has no antitumor effects, as we demonstrated in the line-10 model by injecting PEG without IL-2 according to the 3-times-weekly schedule (data not shown).

No cure was obtained with IL-2 treatment in this study, nor has any cure of the line-10

tumor with IL-2 treatment been reported [27]. Although it cannot be excluded that locoregional IL-2 application with increased frequency and at higher doses might cure guinea-pigs with established tumors, this would not undermine the superiority of PEG-IL-2. A very successful local immunotherapy approach in the line-10 guinea-pig tumor has been described before using Bacillus Calmette-Guérin (BCG). One intratumoral BCG injection on day 7 completely inhibited tumor growth, and resulted in protecting systemic anti-line-10 immunity from 1 week after BCG injection [28].

An important finding in this study is the significance of the intratumoral injection route. As on day 7 tumor cells are present both at the inoculation site and in the regional lymph nodes, in the first experiments both sites were treated. However, subsequent tests indicated that the intratumoral PEG-IL-2 injection was principally, and maybe even completely, responsible for the antitumor effect. There is a tendency for the combination of intratumoral and perilymphatic PEG-IL-2 administration to suppress lymph-node metastases more efficiently (compare treatment groups 12 and 14, Table 1); this point certainly deserves further investigation. On the other hand, it was clearly proven that merely perilymphatic PEG-IL-2 administration is of no benefit in this model, even after surgical resection of the primary tumor. The advantage of the intratumoral administration route is difficult to explain. IL-2-stimulated tumor recognition by the host immune system directly at the tumor site might play a role [29]. However, the regional lymph nodes also contained tumor cells when treatment was started. On the other hand, the intratumoral route might provide an efficient way of reaching the draining lymph nodes. This is suggested by the very fast transport of blue dye to the regional lymph nodes after intratumoral injection of Patent Blue V (personal observation, in head and neck cancer patients). Our finding concerning the injection site agrees with that of Vaage [14,26] who found that therapeutic effects resulted from IL-2 alone or IL-2 liposome-gel preparations injected directly next to, but not 2-3 cm from, the tumor. However, it is inconsistent with the clinical results of Cortesina *et al.* [17], who described effects of perilymphatic IL-2 injections in recurrent head and neck cancer, which we could not confirm in patients with primary advanced disease [30].

In conclusion, PEG-IL-2 appears to be a valuable substance for intratumoral immunotherapy. Repeated injections given 3 times weekly at intermediate doses result in cure of established tumors with micrometastases in guinea-pigs, without signs of toxicity. A role for additional perilymphatic injections cannot be totally excluded and therefore deserves further study. Even so, the capacity of intratumoral PEG-IL-2 therapy started at later stages of disease has to be evaluated. The present results should be considered in the development of clinical studies, for example in head and neck cancer.

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Chapter 6

Histological and immunohistochemical analysis of line-10 tumor regression induced by intratumoral PEG-interleukin-2 therapy

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Summary

Background: Guinea-pigs with palpable line-10 tumors on the flank and regional lymph-node micrometastases were cured by intratumoral injections of polyethylene glycol-modified interleukin-2 (PEG-IL-2), and developed protective systemic antitumor activity.
Methods: To explore the working mechanisms involved serial histological and immunohistochemical analysis of tumors and tumor-draining lymph nodes from PEG-IL-2-treated and control animals was performed.
Results: Before starting treatment $CD4^+$ T cells were observed in close contact with the tumor. Apparently gradual tumor regression was accompanied by an intense inflammatory reaction with T cells, eosinophils, macrophages, and fibroblasts, surrounding and compartmentalizing the tumor. During treatment CD8-like $CT6^+$ T cells infiltrated into the substance of the tumor. Expression of major histocompatibility (MHC) class I molecules by the tumor cells as detected with immunohistochemical methods was low.
Conclusions: This study does not permit certain conclusions about the working mechanism of intratumoral PEG-IL-2 therapy. An antitumor immune response might be elicited by direct activation of the $CD4^+$ T cells at the tumor site by the injected PEG-IL-2. The infiltration of CD8-like $CT6^+$ T cells into the tumor suggests a cytotoxic T cell effector function. In this respect the observed low MHC class I expression by the tumor is discordant. The role of the eosinophils, macrophages and fibroblasts remains speculative. A concerted action of different types of immune-competent and inflammatory cells might be responsible for the obtained antitumor reaction.

Introduction

Interleukin-2 (IL-2) is naturally secreted by T cells upon antigenic activation and has an essential role in the generation of an immune response [1]. Peritumoral supply of IL-2 in experimental tumors from the moment of subcutaneous inoculation was shown to elicit an antitumor immune response, which resulted in suppression of tumor growth [2-6]. Functional analysis indicated that both specific T cells and aspecific immune mechanisms were involved in this response [4-7]. We studied the therapeutic effects of locoregional injections with IL-2 or polyethylene glycol-modified IL-2 (PEG-IL-2) in established malignant disease. For this purpose guinea-pigs with palpable intradermal line-10 tumors and micrometastases in the tumor-draining lymph nodes were used [8,9]. We previously described that cure of these animals could be obtained with PEG-IL-2 injections, in which the intratumoral administration route was obligatory [9]. Repeated experiments indicated that intratumoral injections with 200,000 U of PEG-IL-2, given 3 times a week for 5 weeks, caused complete tumor regression with suppression of metastatic lymph-node growth and long-lasting disease-free survival in 80% and 40% of the animals, when therapy was started on day 7 or day 14-28 after tumor-cell inoculation, respectively. Cured animals had protective immunity against line-10 rechallenge [9], which was transferable to naive guinea-pigs by spleen lymphocytes [10]. Furthermore, the PEG-IL-2-induced antitumor effect was completely abrogated by pretreatment of the animals with anti-thymocyte serum. This suggests that T lymphocytes are involved in the antitumor effect. However, we could not demonstrate anti-line-10 cytotoxicity of lymphocytes isolated from the tumor, tumor-draining lymph node, or spleen of PEG-IL-2-treated animals *in vitro* [10]. In this paper we describe serial histological and immunohistochemical analysis of regressing line-10 tumors and lymph-node metastases. This study was performed to shed more light on the starting-point and the mechanism of effective intratumoral PEG-IL-2 therapy. Immune-cellular infiltration at the tumor site was studied with emphasis on T lymphocytes. As these cells interact with other cells in a major histocompatibility complex (MHC)-dependent way, also MHC expression on the tumor cells was determined.

Materials and methods

Animals

Female Sewall-Wright inbred strain-2 guinea-pigs were obtained from the National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD, and used when 2 to 3 months old, weighing 400 to 600 g.

Tumor

The line-10 tumor is syngeneic to strain-2 guinea-pigs. This hepatocellular carcinoma was originally induced by oral administration of diethylnitrosamine, and converted to the ascites form [8,11]. The tumor-cell line was maintained as frozen stock, and propagated intraperitoneally. Intradermal inoculation with 1×10^6 cells on the flank results in local tumor growth, axillary lymph-node metastases within 7 days, and lung metastases later on. Tumor cells were used in their 12th passage.

Therapeutics

Recombinant human IL-2 modified by the covalent attachment of 2-3 PEG M_r 7,000 molecules per IL-2 molecule (PEG-IL-2) was provided by Eurocetus, Amsterdam, The Netherlands. PEG-IL-2 has a higher molecular weight, comparable *in vitro* activity, and a plasma clearance that is 10-fold lower than that of rIL-2 [12]. The specific activity of PEG-IL-2 was 9.4×10^6 U/mg IL-2 protein. Solutions containing 1×10^6 U/ml were prepared from lyophilized PEG-IL-2 by reconstitution in sterile water and dilution in phosphate-buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA), and stored at -20°C until use.

Experimental design

Guinea-pigs were injected intradermally in the left anterior flank with 1×10^6 line-10 tumor cells on day 0. Intratumoral injections with 200,000 U PEG-IL-2 were started on day 7, and given 3 times weekly for 5 weeks. Control animals received injections with PBS containing 0.1% (w/v) BSA. Tumors and first tumor-draining axillary lymph nodes were obtained from guinea-pigs killed prior to treatment (day 7), and on days 14, 28 and 42 during PEG-IL-2 or PBS+BSA treatment (n=4 per group). One half of each tumor and lymph node was fixed in formalin and embedded in paraffin, and the other half was frozen immediately into liquid nitrogen. As a control for tumor-independent PEG-IL-2-induced changes, injection sites and regional lymph nodes from 4 nontumor-bearing guinea-pigs, given intradermal 200,000 U PEG-IL-2 injections on the flank 3 times weekly for 3 weeks, were excised.

Immunohistochemistry

Cryostat tumor sections were stained with a 2-step immunoperoxidase procedure as described before [13], using the following monoclonal antibodies (MAbs): anti-guinea-pig CD4 rat MAb H155 [14], recognizing helper T cells; the putative anti-guinea-pig cytotoxic/suppressor T cell mouse MAb CT6 [15,16]; mouse MAb MR1 [17], reactive with guinea-pig monocytes and macrophages, but not with Langerhans' cells in the skin;

mouse MAb MSgp4, reactive with guinea-pig MHC class I [18]; mouse MAb 27E7, reactive with guinea-pig MHC class II [19]; anti-human keratin 5/8 MAb OVTL 12-5, reactive with all line-10 tumor cells [20]. All MAbs were kind gifts of the originating laboratories. As secondary antibodies in the immunoperoxidase procedure peroxidase-conjugated rabbit-anti-mouse Ig and rabbit-anti-rat Ig were used. The peroxidase label was visualized by incubation with a 3,3'diaminobenzidine tetrahydrochloride solution as a substrate. PEG-IL-2-treated tumors on days 28 and 42 showed marked staining due to endogenous peroxidase activity of infiltrating eosinophils. For this reason an immuno-alkaline phosphatase method was used on these sections as well. After incubation with alkaline phosphatase-conjugated rabbit-anti-mouse Ig or rabbit-anti-rat Ig as the secondary antibody, the alkaline phosphatase label was visualized by incubation with Fast Red substrate solution. Negative controls were obtained by incubation with PBS+BSA without the primary antibody. Positive controls included normal spleen and lymph-node sections.

Evaluation

Histopathological analysis on routinely fixed paraffin sections included pathological diagnosis, growth pattern, mean number of mitotic figures per high power field (HPF, 400×), lymphatic vessel invasion, necrosis, and vascularization. At the tumor site, overall intensity of leukocytic infiltrate, and intensity of eosinophils (HE-stained paraffin sections), and intensity of T lymphocytes and macrophages (immunohistochemically stained frozen sections) were scored according to the following scales: (1) tumor-surrounding tissue: –, no or only very few cells; +, scattered cells; ++, cells in groups; +++, cells in a continuous rim surrounding tumor islets; and (2) within the tumor substance: –, no or only very few cells; +, scattered cells all over the tumor mass; ++, strong intensity of infiltrating cells. Expression of MHC class I and II antigens on tumor cells was estimated as percentage of the total number of tumor cells in the slide, and scored in 1 of 5 categories: 0-5%; 6-25%; 26-50%; 51-75%; and 76-100%. MHC class II expression was not useful as an activation marker for lymphocytes, because of the constitutively high expression on guinea-pig T cells, as described before [16].

Results

Histology of primary tumors

Histopathological analysis of the intradermal growing line-10 tumor on HE-stained paraffin sections showed a pleomorphic undifferentiated malignant tumor with a high (10-20/HPF) mitotic frequency (Fig. 1). On day 7, prior to therapy, small tumor cell nests in

the deep dermis and subcutaneous fat were intermingled with an intense, predominantly lymphocytic infiltrate.

In the PBS+BSA-treated guinea-pigs a massive layer of tumor cells with a mean diameter of 8 mm had developed on day 14. Lymphatic vessel invasion was observed. The tumors were separated from the skin muscle by a narrow but continuous border of mononuclear cells. On day 28 the tumors had expanded laterally in the subcutaneous fat, and invaded the tendinous sheet along the skin muscle. Deep infiltration in the skin muscle was observed on day 42, when the tumor area had reached a size of about 13x5 mm. The tumor stroma then contained few leukocytes (+) and large blood vessels. Nevertheless, focal central ischemic necrosis occurred.

In the PEG-IL-2-treated tumors on the other hand, an impressive inflammatory reaction developed. While on day 14 the tumor area was comparable to the PBS+BSA treated tumors, the border of mononuclear cells along the skin muscle was 2-3 times as broad, and contained lymphocytes, histiocytes, eosinophils, and fibroblasts. On day 28 the tumor area was compartmentalized by sheets of fibrous stroma, containing mononuclear inflammatory cells with a granulomatous aspect. Besides many histiocytes and eosinophils (++/+++, Table 1), also fibroblasts, multinucleated giant cells, and neutrophilic granulocytes were observed, while lymphocytes were identified only sparsely in the HE-stained sections (Fig. 1). This inflammatory reaction widely spread through the dermis, and within and beyond the skin muscle. On day 42 also phagocytic cells with large vacuoles were observed in the tumor-surrounding stroma. The tumor area in the PEG-IL-2-treated tumors had decreased on day 28, and on day 42 in 3 samples 1-4 small islets (<1 mm^2 each; Fig. 2a) with tumor cells remained, while in the fourth sample no tumor cells could be detected, neither on HE-stained sections, nor with OVTL 12-5 MAb staining. In the regressing tumors, individual tumor cell death was observed along the borders of the tumor cell islets, especially on day 42 (Fig. 2a,b). In the sample from day 28 with the largest tumor area, more extensive tumor decline was observed with subtotal central tumor necrosis, leaving less than 10% viable tumor cells along the borders. The necrotic area in this tumor was heavily infiltrated by neutrophilic granulocytes. Changes in blood vessels such as occlusion, clotting or endothelial swelling were not observed. The frequencies of mitotic figures in PEG-IL-2- and PBS+BSA-treated tumors were comparable, except for day 42, when the tumor areas in the PEG-IL-2-treated tumors, however, were too small for adequate comparison.

Table 1. *Intensity of tumor-surrounding eosinophils, macrophages, and T cells in PEG-IL-2-treated and control intradermal growing line-10 guinea-pig tumors.*

Intensity	Eosinophils[a]				Macrophages[b]			
	day 7	14	28	42	day 7	14	28	42
+++			●●	●●●				●
++			●●	■		●●	●●●	●■■
+		●●	○○	○○○	○○○○	●●○○○	○○	○○○○
−	○○○○	●●○○○	○○	○				

Intensity	Cytotoxic/suppressor T cells[c]				Helper T cells[d]			
	day 7	14	28	42	day 7	14	28	42
+++			●●	●●			●●	●●
++		●		■■	○○○○	●●●●○○	○	■
+	○○○○	●●○○○	●○○○	○○○○		○○	●○○	■○○○○
−		●○						

Detected in HE-stained paraffin sections[a], or frozen sections stained with MAbs MR1[b], CT6[c], or H155[d].
○, control tumor; ●, PEG-IL-2-treated tumor; ■, PEG-IL-2-treated sample, in which no tumor cells could be detected.
On day 28, only 3 frozen samples per group were available.
Intensity scored as: −, no or only very few positive cells; +, scattered positive cells; ++, positive cells in groups; +++, positive cells in a continuous rim surrounding tumor islets.

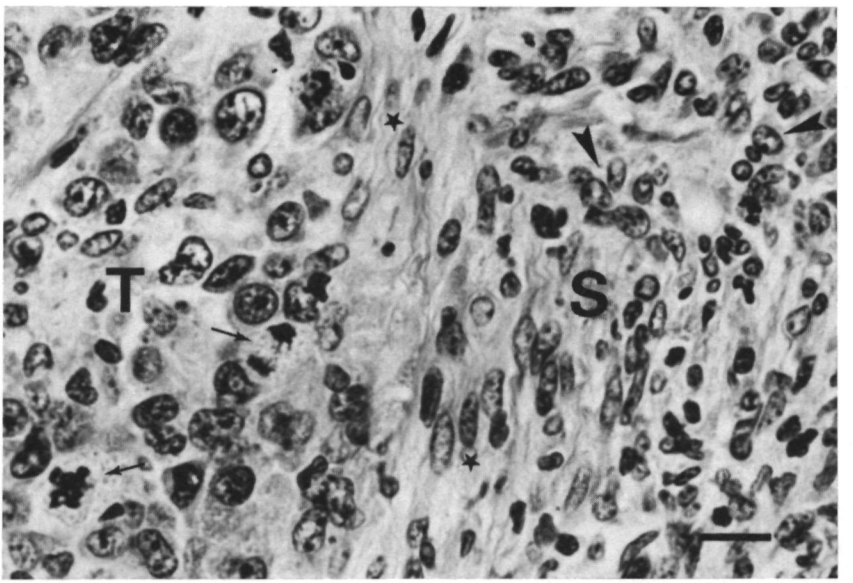

Figure 1. Peripheral area of a PEG-IL-2-treated line-10 tumor on day 28 after inoculation, HE-stained paraffin section. T, tumor mass, with mitotic figures (arrows); S, tumor-surrounding stroma, containing histiocytes (arrowheads) and fibroblasts (*). Bar = 25 µm.

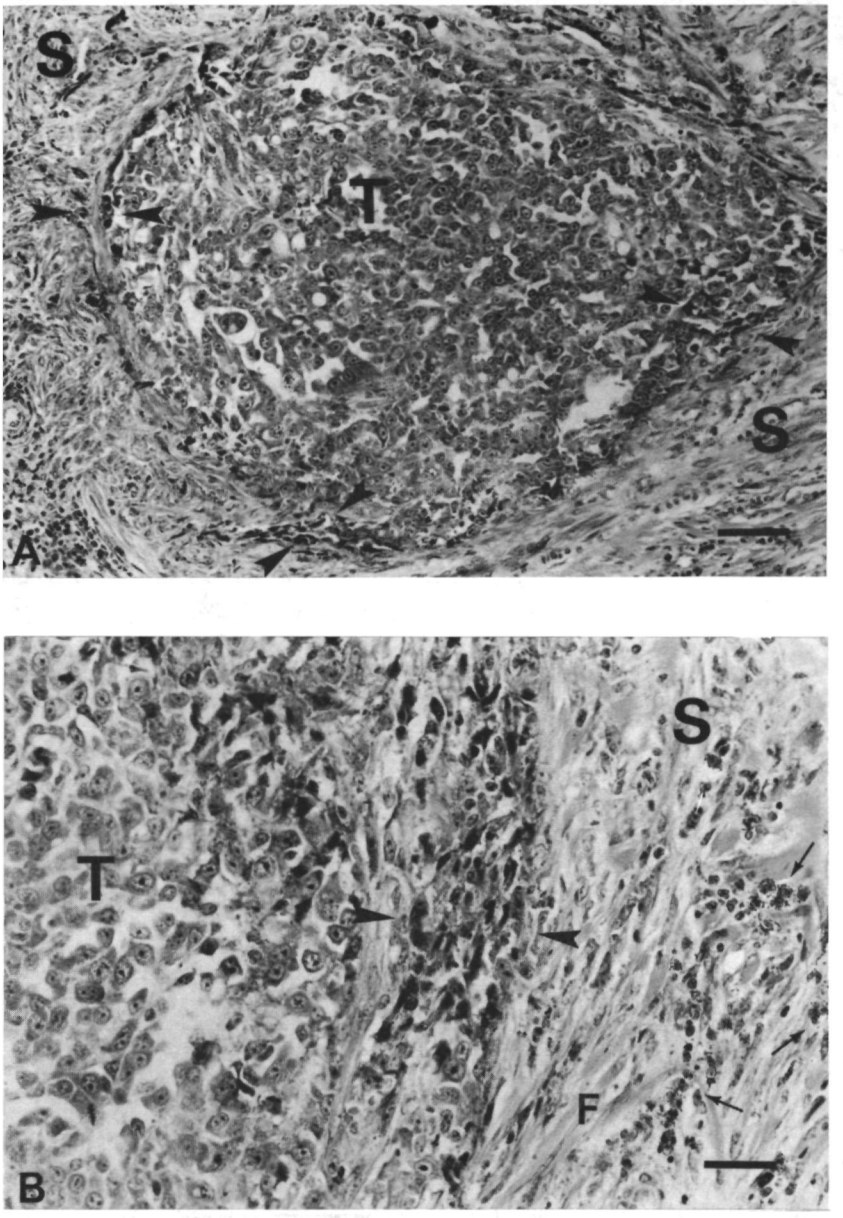

Figure 2. Paraffin sections of PEG-IL-2-treated line-10 tumors on day 42 after inoculation showing a small tumor islet (T) completely surrounded by stroma (S) with inflammatory reaction. (*a*) Bar = 100 μm, (*b*) Bar = 50 μm. A rim of necrotic tumor cells is observed at the edge of the tumor (arrowheads). The stroma shows fibrosis (F), and the May-Grünwald-Giemsa staining used highlights the large number of eosinophils (arrows).

Regional lymph nodes

Table 2 shows size and metastatic load of the tumor-draining lymph nodes, assessed on HE-stained paraffin sections, and frozen sections stained with MAb OVTL 12-5. Tumor cells were seen in the marginal sinus from day 7. In PBS+BSA-treated animals large metastases developed, almost completely replacing lymphatic tissue on day 42.

In comparison with the PBS+BSA-treated animals no clear cortical or paracortical hyperplasia occurred in the lymph nodes of PEG-IL-2-treated animals. However, these lymph nodes became diffusely infiltrated with eosinophils, and showed a remarkable reaction on the edge. The lymph-node capsules became broadened, which culminated on day 28 in a wide lymph-node-surrounding zone containing mainly eosinophils and

Table 2. *Regional lymph-node metastases from intradermal line-10 tumors in PEG-IL-2-treated and control guinea-pigs.*

Lymph-node area (mm^2)[a]					Metastatic load[b]				
	day 7	14	28	42		day 7	14	28	42
>100				○○○○	+++			○	○○○○
51-100					++			○○○	
26-50			○○	●●	+	○	● ○○○○	●	●[c]
13-25	○	○	●●● ○○	●●	±		●●	●●	●
≤12	○○○	●●●● ○○○	●		−	○○○	●	●	●●

○, control tumor; ●, PEG-IL-2-treated tumor.

[a] Multiplication of largest diameters on section, not including the perilymphatic inflammation observed in PEG-IL-2-treated animals.

[b] Based on histopathological analysis of HE-stained paraffin and frozen sections, and on frozen sections stained with MAb OVTL 12-5. —, no tumor cells; ±, tumor cells identified on OVTL 12-5 stained sections only (<10 cells per slide); +, invasion of marginal sinus and/or few tumor cells scattered in lymph node; ++, tumor involves 25-75% of the lymph-node area; +++, tumor involves >75% of lymph-node area.

[c] Excluding 90% tumor necrosis.

histiocytes (Fig. 3), and resembling the inflammatory reaction induced in the stroma of the intradermal tumor. No specific reaction was observed in the immediate vicinity of the viable micrometastases within the lymph nodes of PEG-IL-2-treated animals on days 14 and 28. On day 42, lymph nodes from 2 PEG-IL-2-treated animals were small, and one of these contained few tumor cells. The remaining 2 were clearly enlarged (26-50 mm^2, Table 2). One showed a large metastasis, which, unlike in PBS+BSA-treated animals, contained 90% dead tumor cells. The other did not show tumor cells, but large fields of histiocytes with centrally some necrosis.

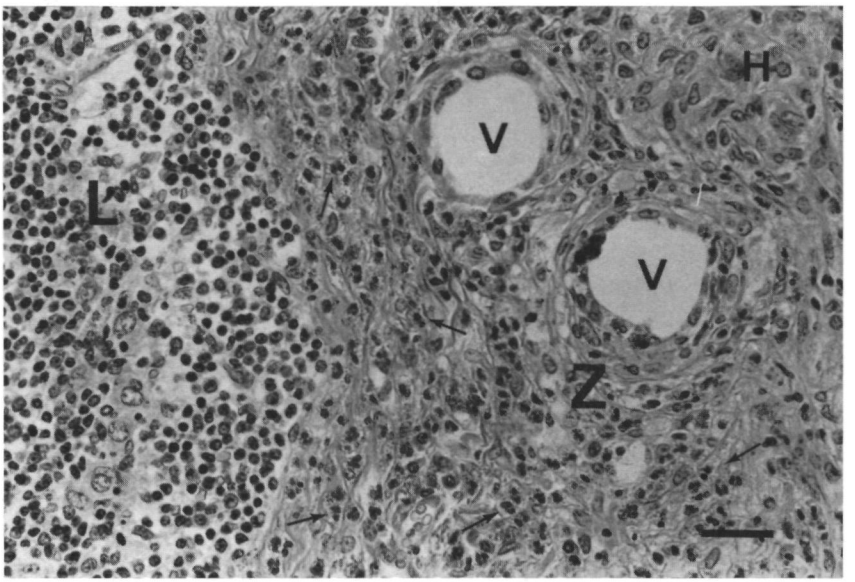

Figure 3. Peripheral part of a lymph node draining a PEG-IL-2-treated Line-10 tumor, obtained on day 28 after inoculation. The lymph node (L) is surrounded by a wide zone (Z) with an inflammatory reaction containing aggregates of histiocytic cells (H), many eosinophils (arrows) and slight fibrosis. V, lipid vacuole surrounded by histiocytes. HE-stained paraffin section. Bar = 50 µm.

Immunohistochemical analysis of the mononuclear cell infiltrate

The intensity of T cell infiltration in the tumor-surrounding stroma as scored with MAbs CT6 and H155 is represented in Table 1. The lymphocytic infiltrate on day 7 mainly consisted of helper T cells. In the PEG-IL-2-treated tumors the stroma surrounding the tumor islets on days 28 and 42 proved to contain considerably more T lymphocytes than identified in the HE-stained sections, with helper T cells (H155$^+$) and cytotoxic/suppressor T (CT6$^+$) cells in about equal numbers. Adjacent tissues, including the skin muscle, were diffusely infiltrated with T cells (mainly CT6$^+$) as well. Within the tumor mass (Table 3), on days 7 and 14 H155$^+$ and CT6$^+$ cells were sparse (scored as –). On day 28, however, within the PEG-IL-2-treated tumors T cells were scattered all over the tumor (Fig. 4), with CT6$^+$ cells outnumbering H155$^+$ cells.

From day 7 MR1$^+$, MHC class II-expressing (27E7$^+$), dendritic cells were evenly spread within the tumor (+/++, data not shown). The tumor-surrounding stroma also contained a considerable number of MR1$^+$ macrophages (+), that increased during treatment (++, Table 1). The vacuolized phagocytic cells observed around PEG-IL-2-treated tumors on day 42 were MR1$^+$.

Table 3. *Intensity of T cell infiltrate within the tumor substance in PEG-IL-2-treated and control intradermal line-10 guinea-pig tumors.*

Intensity	Cytotoxic/suppressor T cells[a]				Helper T cells[b]			
	day 7	14	28	42	day 7	14	28	42
++			●●					
+			●	●●			●●	
–	○○○○	●●●● ○○○○	○○○	○○○○	○○○○	●●●● ○○○○	● ○○○	●● ○○○○

Immunohistochemically detected in frozen sections with MAbs CT6[a] and H155[b].

○, control tumor; ●, PEG-IL-2-treated tumor. On day 28, only 3 frozen samples per group were available. On day 42, no tumor was found in the frozen sections from 2 PEG-IL-2-treated animals, whereas scoring is based on a very small residual tumor area in the other 2.

–, no or almost no positive cells; +, scattered positive cells all over the tumor mass; ++, strong intensity of infiltrating cells.

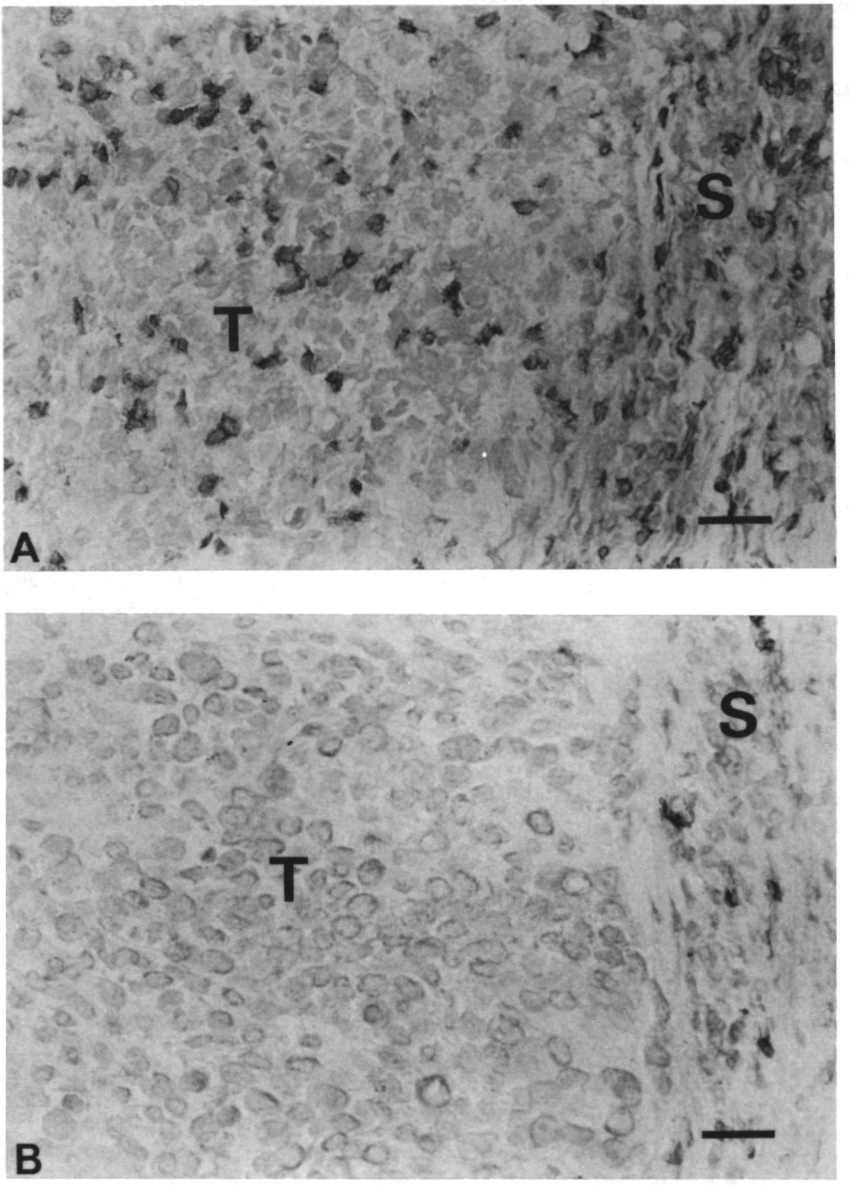

Figure 4. Immunoperoxidase staining with MAb CT6 directed against cytotoxic/suppressor T cells on frozen sections of line-10 tumors on day 28 after inoculation. In the PEG-IL-2-treated tumor (*a*) an intense infiltration of stained cells is observed both in the tumor-surrounding stroma (S) and within the substance of the tumor (T). In the PBS+BSA-treated tumor (*b*) few stained cells are seen in the stroma only. Bar = 50 μm.

MHC expression on tumor cells

The immunohistochemically detected expression of MHC class I and II antigens on line-10 tumor cells was low. In most tumors 6-25% of the cells, situated in the periphery of the tumor, showed weak to moderate staining with anti-MHC class I MAb MSgp4 (data not shown). This percentage appeared to increase in the PEG-IL-2-treated tumors on day 28. However, it was almost impossible to differentiate between the strongly MHC class I-positive infiltrating cells, penetrating into the tumor, and the tumor cells themselves. Expression of MHC class II as recognized by MAb 27E7 was not observed (<5% of cells), although the borders of PEG-IL-2-treated tumors were not interpretable on days 28 and 42 because of the strongly positive tumor-surrounding inflammatory cells.

Effects of PEG-IL-2 in nontumor-bearing guinea-pigs

Intradermal PEG-IL-2 injections in nontumor-bearing animals for 3 weeks (comparable to day 28 in PEG-IL-2 treated tumor-bearing animals) resulted in disturbance of the normal subcutaneous fat by intense infiltration with eosinophils (++) and histiocytes (++). Phagocytic cells with large vacuoles were seen, and also some multinucleated cells and fibroblasts. Immunoperoxidase-stained frozen sections showed scattered (+) $CT6^+$ and $H155^+$ T cells at the PEG-IL-2 injection site. In contrast, no (–) $CT6^+$ and $H155^+$ cells were observed in the normal, noninjected guinea-pig deep dermis and subcutaneous fat. The regional lymph nodes from PEG-IL-2-injected nontumor-bearing guinea-pigs were not enlarged (≤ 12 mm^2), and a minor capsular reaction with eosinophils and histiocytes was observed (data not shown).

Discussion

The antitumor mechanisms induced by either systemic or locoregional IL-2 administration *in vivo* are incompletely understood. Experimental studies on locoregional IL-2 supply mostly concerned growth inhibition of tumor-cell inoculations [2-7]. In contrast, the line-10 tumor used in this study provides a model with palpable tumors, and regional micrometastases, in which 80% cure was obtained with intratumoral administration of PEG-modified IL-2. The present histological and immunohistochemical study indicates that this therapeutical effect is accompanied by an intense inflammatory reaction of mixed composition, surrounding and compartmentalizing the tumor area. Such analysis in one tumor model does not allow conclusions on the working mechanism of IL-2 *in vivo*. The shortage of suitable monoclonal antibodies for immunohistochemical analysis of guinea-pig tissues constitute a special limitation of this model. Nevertheless, a study like this may

provide background information for the interpretation of therapeutical and functional immunological studies, and leads for further research. Several aspects will be discussed in detail.

We and others found that the peri- or intratumoral administration route was necessary to cure tumor-bearing animals with IL-2 and to induce immune memory [2,3,9]. Analysis of the line-10 tumor site prior to starting PEG-IL-2 therapy showed tumor cells in close contact with T cells which were predominantly $CD4^+$. It is conceivable that PEG-IL-2 directly activated these tumor-sensitized helper T cells to initiate an immune response and to induce immune memory. Requirement of lymphocytes at the tumor site for locoregional IL-2 therapy to be effective was also described in other models [2,3]. In studies on the *in vivo* growth of tumor cells engineered to produce IL-2 by IL-2 gene transfection, it was shown that $CD4^+$ T cells were essential for the induction of immune memory [5], whereas $CD8^+$ T cells were required for tumor rejection [4-6]. That $CD8^+$ T cells were involved in PEG-IL-2-induced tumor regression in our model as well, was suggested by the observed infiltration of CD8-like $CT6^+$ cytotoxic/suppressor guinea-pig T cells into the regressing tumors. No other inflammatory cells penetrated within the substance of the tumor as deeply as these cells did. On the other hand, the immunohistochemically low MHC class I expression on line-10 tumor cells is not in favor of interaction with MHC class I-restricted $CD8^+$ T cells. The absence of *in vitro* anti-line-10 cytolytic activity of tumor-, lymph-node-, and spleen lymphocytes from PEG-IL-2-treated animals, described earlier [10], in itself does not exclude a role for $CD8^+$ T cells, as others showed that also noncytolytic $CD8^+$ tumor-infiltrating lymphocytes mediated tumor regression *in vivo* [21].

Besides lymphocytes, an intense inflammatory reaction with eosinophils, macrophages and fibroblasts, both in the tumor-surrounding stroma and around the tumor-draining lymph node, accompanied the PEG-IL-2-induced line-10 regression. A similar but less intense reaction was observed after intradermal PEG-IL-2 injections in nontumor-bearing animals. The question arises whether these eosinophils, macrophages and fibroblasts contribute to the PEG-IL-2-induced therapeutical effect. The local inflammatory reaction probably is not specific for PEG-IL-2. In humans intradermal injections with unmodified IL-2 induced local infiltration of $CD4^+$ and $CD8^+$ T cells, and monocytes, reminiscent of a delayed-type hypersensitivity (DTH) reaction to antigen [22]. Infiltration of eosinophils [7] and macrophages [4,23] at the tumor site following local supply of IL-2 was also described before. IL-2 was shown to display direct chemoattractant effects on eosinophils, which express functional IL-2 receptors [24]. On the other hand, the peripheral-blood eosinophilia described in patients receiving systemic IL-2 therapy [25], was found to be mediated by IL-5 release from IL-2-stimulated T lymphocytes [26,27]. Recently, Rivoltini *et al.* [28] described *in vivo* IL-2-activated peripheral-blood eosinophils of cancer patients

:o possess direct cytotoxicity and antibody-dependent cytotoxicity (ADCC) against ıllogeneic tumor cells *in vitro*. Tepper *et al.* [29] found IL-4-induced antitumor effects to ɔe dependent on local eosinophil infiltration. Otherwise, IL-5-induced eosinophilic in-ilıration at the tumor site in itself did not induce an antitumor response [30]. In a lifferent model, an antitumor effector role for eosinophils in co-operation with neutrop-ıils, macrophages and lymphocytes was suggested in an electron-microscopic study on IL-l-induced rejection [31], since these inflammatory cells were observed in close contact ıvith the tumor cells and with each other. The latter is in contrast to our results, as we ɔbserved eosinophils in the tumor-surrounding stroma and tissues, but not directly adja-ɔent to the tumor cell islets. Eosinophils may interact with other immune cells by cytokine ɔroduction, and MHC class II-dependent antigen presentation to $CD4^+$ T cells [32].

The accumulation of macrophages around the tumor islets we observed, together with T ɔells and the peritumoral fibrotic reaction, resembles the fibrous tumor encapsulation lescribed by Steerenberg *et al.* [13] in line-10 tumor regression induced by intratumoral 3acillus Calmette-Guérin (BCG) injection, and also by Vaage [23] in local IL-2 therapy of nouse mammary carcinomas. Macrophages, triggered by lymphokines from helper T cells, ɔan produce cytokines which induce fibroblast proliferation and collagen synthesis [23,33]. Such immunologically enhanced fibrous encapsulation in itself might be beneficial by con-ɔolling tumor growth [34]. The macrophages we observed around regressing tumor-cell ıests, and in large fields within one of the lymph nodes of day 28, suggested a role in the ɔhagocytosis of tissue debris and scavenging. Direct tumoricidal activity was described in IL-2-stimulated monocytes [35].

In the present study, a gradual decrease of the line-10 tumor mass was observed, with ndividual tumor-cell death along the borders of the tumor islets. In 2 samples out of the ɔtal group of tumors and lymph nodes analyzed, however, extensive central tumor cell leath was observed. This overwhelming reaction might be related to tumor-growth rate, as ɔoth samples contained a relatively large tumor area on day 28-42 after inoculation. Extensive IL-2-induced tumor necrosis was also described in the fast-growing mouse SL-2 ymphoma [36].

In this study it was shown that cure of established line-10 guinea-pig tumors induced by ıntratumoral PEG-IL-2 injections is accompanied with an intense locoregional inflam-ıatory reaction. The obtained results are compatible with a role for $CD4^+$ T cells in the ınduction phase of the immune response, and are suggestive for an effector function of CD8-like $CT6^+$ T cells. In the latter the low MHC class I expression by the tumor is liscordant. The role of eosinophils, macrophages and fibroblasts remains speculative, and leserves further analysis. A concerted action of different types of immune-competent and ınflammatory cells might be responsible for the obtained antitumor reaction.

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Chapter 7

Intratumoral PEG-interleukin-2 therapy in patients with locoregionally recurrent head and neck squamous-cell carcinoma

Submitted for publication as:

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This study was presented at the 84th annual meeting of the American Association for Cancer Research, May 1993, Orlando, Florida (*Proc AACR* 1993; 34:218).

Background: Immunotherapy with local supply of interleukin-2 (IL-2) has proven enhanced efficacy compared to systemic administration in several experimental tumor models. We previously showed that intratumoral injections with polyethylene glycol-modified IL-2 (PEG-IL-2) could cure guinea-pigs with palpable tumors and regional micrometastases. In the present study we assessed the feasibility and antitumor effects of this PEG-IL-2 treatment schedule in a clinical situation.

Patients and methods: Nineteen patients with locoregionally recurrent head and neck squamous-cell carcinoma (HNSCC), not exceeding 8 centimeter as largest diameter, and for whom no curative treatment was available, were entered. Intratumoral injections with 200,000 U of PEG-IL-2 were given 3 times weekly in courses of 4 weeks into 11 local and 11 regional tumor recurrences.

Results: Treatment was given on an out-patient basis, and was well tolerated. Temporary regional swelling and redness developed in 8 patients. In 7 of these patients systemic eosinophilia was documented. Low-grade fever occurred in 1 patient. Median duration of treatment was 4 weeks (range 2-14 weeks). Seventeen patients were evaluable for response. One complete response (CR; 6%; duration 91 weeks), and 6 stable diseases (SDs; duration 8-57+ weeks) were recorded. The CR and the 3 best SDs (23, 40, 57+ weeks) occurred in patients with a single regional tumor recurrence of relatively small size. During treatment, all 4 developed locoregional edema and redness, and high levels of circulating eosinophils. Median survival was 23 weeks for the total patient group, and >45 weeks for the patients with SD.

Conclusion: Intratumoral injection of PEG-IL-2 in patients with HNSCC is feasible. A local inflammatory reaction may contribute to the observed antitumor effect. This treatment appears beneficial for highly selected patients. The objective response rate is insufficient for wide clinical application.

Introduction

In several experimental tumors the therapeutical effects of repeated locoregional injections with relatively low doses interleukin-2 (IL-2) have been described. Both suppression of tumor growth [1-3], and regression of established tumors [3,4] were reported. Besides these locoregional effects, this approach was shown to induce specific systemic antitumor immunity, resulting in rejection of tumor cells inoculated simultaneously at distant sites [3,4], or subsequent tumor rechallenges [1,5]. The peritumoral administration route appeared to be a prerequisite for these effects [1,4,6], as was the presence of lymphocytes at the tumor site [1,6], and an immunocompetent host [7]. It was hypothesized that host immune cells at the tumor site were activated by exogenous IL-2 to produce cytokines, and to recruit specific and nonspecific immune effector mechanisms [7].

Head and neck squamous-cell carcinoma (HNSCC) appears a suitable disease to evaluate the feasibility and efficacy of local IL-2 immunotherapy in man. The tumor is localized near the body surface, metastasizes primarily to the neck lymph nodes, and tends to recur locoregionally after primary surgery and/or radiotherapy. Primary tumors are generally infiltrated with T lymphocytes, which have been shown to obtain cytotoxic activity after activation by IL-2 *in vitro* [8]. Moreover, extensive submucosal and nodular lymphatic tissue is localized in the direct vicinity. Although locoregional, probably tumor-induced, immunosuppression in HNSCC is well-documented [9], cytotoxic T cells specific for HNSCC targets were obtained from tumor-draining lymph nodes both by activation with IL-2 *in vitro* [10], and by locoregional IL-2 application *in vivo* [11].

The therapeutic effects of locoregional IL-2 administration in HNSCC have been studied in several ways. Perilymphatic injection (*i.e.*, around the tumor-draining lymph nodes) was first described by Cortesina *et al.* [12]. With 10 daily low-dose IL-2 injections, they initially obtained 3 complete responses (CR; duration 4-6 months) and 3 partial responses (PR) in 10 patients with locoregionally recurrent HNSCC [12]. This high rate of objective responses, however, was not confirmed by the same authors when enlarging their study group [13], nor by us in patients with locoregionally far-advanced primary disease [14]. PR were also described in studies using combined perilymphatic and intratumoral IL-2 injections (2/36 patients) [15], intra-arterial IL-2 infusion (2/12 patients) [16], and combined locoregional IL-2 and LAK cell administration (3/6 and 3/14 patients, respectively) [17,18].

In the syngeneic guinea-pig line-10 tumor model, characterized by fast-growing regional lymph-node metastases, we analyzed locoregional IL-2 therapy, using polyethylene-glycol-modified IL-2 [19]. As compared to unmodified IL-2 this PEG-IL-2 has enhanced

solubility and plasma half-life [20,21], and increased *in vivo* antitumor activity in experimental tumors after intravenous (i.v.) administration [20,22]. Applying PEG-IL-2 locoregionally, 3 times weekly, we could cure guinea-pigs with palpable line-10 tumors on the flank and micrometastases in the regional lymph nodes. Furthermore, protective anti-line-10 activity was induced [19]. Intratumoral, and not perilymphatic, administration was a prerequisite for this effect. Based on these experimental results, we developed the present clinical study for patients with locoregionally recurrent HNSCC. The main aims were to evaluate the feasibility and the local antitumor effects of intratumoral PEG-IL-2 injections in HNSCC, in a schedule which we found to be optimal in the animal study. As patients with extended tumor growth were not likely to benefit from this approach, the tumor size was restricted arbitrarily. PEG-IL-2 has been applied before i.v. in cancer patients in phase I [21] and II [23] studies, and intradermally in human immunodeficiency virus (HIV)-type 1-infected patients [24], but not locoregionally in cancer patients. To learn more about the locoregional effects, tumor biopsies were scheduled to be taken before and after PEG-IL-2 therapy whenever possible, to study the mononuclear cell infiltrate, and human leukocyte antigen (HLA) expression on the tumor cells.

Materials and methods

Patients

Eligible patients met the following criteria: measurable, histologically or cytologically confirmed locoregional recurrence of HNSCC, for which no curative treatment was available, and newly occurring after previous surgery and/or radiotherapy. No more than two tumor sites were allowed. Tumor recurrences had to be accessible to injection, and should not exceed 8 cm as largest diameter. Furthermore, patients should have a Karnofsky performance status of 80% or more, an expected survival of 3 months at least, normal hematologic parameters, adequate hepatic, renal, and cardiac function, no other significant medical conditions requiring ongoing therapy, no organ allografts, and no distant metastases. Concurrent corticosteroid therapy was not permitted. Between November 1991 and May 1993, 19 patients gave their informed consent and were entered in this study, at the University Hospitals of Nijmegen (n=11), Utrecht (n=5), Maastricht (n=2), and Düsseldorf (n=1). Patient characteristics are outlined in Table 1.

Table 1. *Patient characteristics*

No. of patients enrolled		19
Males/females		13/6
Median age, years (range)		69 (46-83)
Karnofsky Performance status		
100%		4
90%		7
80%		7
60%		1[a]
Primary tumor site		
Oral cavity		6[b]
Larynx		6
Oropharynx		4
Hypopharynx		1
Vestibulum nasi		1
Ear		1
Prior therapy for HNSCC		
Radiotherapy		19
Surgery primary tumor		14
Neck lymph node dissection		
- unilateral		6
- bilateral		5
Chemotherapy		4
Median time between finishing previous therapeutic intervention and diagnosis of current tumor recurrence, months		4
Range:	1.5-4 months	10
	4.5-12 months	7
	12-24 months	0
	>24 months	2

[a] As this Karnofsky score was caused by decreased mobility due to a cerebrovascular accident years before, the patient was still accepted for this study.

[b] Including 2 patients with a history of multiple oral carcinomas.

Treatment

Recombinant human IL-2 modified by the covalent attachment of 2-3 PEG M, 7,000 molecules per IL-2 molecule (PEG-IL-2) was provided by Eurocetus, Amsterdam, The Netherlands. The PEG-IL-2 used had a specific activity of 85.7×10^5 (Lotno. LCP-920) or 40.0×10^5 (Lotno. LCP-039B) U/mg IL-2 protein. The treatment regimen involved intralesional injections with 200,000 U PEG-IL-2 in 0.5 ml normal saline containing 0.1% human serum albumin. Injections were given 3 times a week (Monday, Wednesday, and Friday) for 4 weeks. If patients had 2 tumor recurrence sites, these were injected simultaneously with 200,000 U each. After 4 weeks, patients were evaluated for clinical response. Patients with progressive disease (PD) were removed from the study. In case of stable disease (SD) treatment was continued till 8 weeks. Objective responders were scheduled to receive 12 weeks of treatment without interruption, unless CR was achieved earlier.

During therapy, changes in performance status and locoregional adverse effects were recorded every treatment day. Hematological parameters were assessed every 2 weeks, and biochemical parameters of hepatic and renal function every 4 weeks. A Chest X-ray was performed before and at the end of PEG-IL-2 treatment.

Assessment of response

During treatment, tumor sizes were measured every 4 weeks by physical examination, ultrasound, or CT scan, as appropriate. CR was defined as disappearance of all evidence of disease for at least 4 weeks, and PR as 50% or greater reduction of the product of the greatest perpendicular tumor diameters for at least 4 weeks. PD was defined as 25% or greater increase in the size of the tumor lesions, or the appearance of new lesions; SD as disease not meeting the criteria for either CR, PR, or PD. Duration of response was reported from the start of PEG-IL-2 treatment. After stopping treatment, patients with SD, PR, or CR were evaluated monthly during the first 6 months, bimonthly from 6-12 months, and 3-monthly thereafter. Patient survival was determined from the start of PEG-IL-2 treatment.

Tissue specimen and immunohistochemical analysis

Pre-treatment tumor samples were analyzed for histopathological grade, intensity of the mononuclear cell infiltrate, and HLA antigen expression. Post-treatment tumor samples were obtained in 4 patients.

HLA class I and II antigen expression on tumor cells was assessed with immunohistochemical methods on paraffin sections, using anti-HLA class I MAb HC10

and anti-HLA-DR serum, as described before [25]. The intensity of the mononuclear cell infiltrate in the tumor-surrounding tissue was assessed on HE-stained sections. In those tumors from which samples were obtained both before and 4 weeks after PEG-IL-2 treatment, additional staining was performed with MAbs UCHL1 (anti-CD45RO, T cells), L26 (anti-CD-20, B cells), and KP1 (anti-CD68, macrophages).

Anti-IL-2 antibodies

Patient sera were obtained before, after 4 weeks, and at the end of PEG-IL-2 treatment, and deep frozen. Presence of IL-2 binding-antibodies in these sera was studied by an Enzyme Linked Immunosorbent Assay (ELISA), as described before [26]. The ELISA titer was defined as the product of the highest serum dilution with an absorbance below 0.5 and the actual absorbance value. ELISA titers exceeding 10 were defined as positive.

Results

Tumor lesions and administration of PEG-IL-2

Clinical characteristics of the treated lesions are represented in Table 2. Ten patients had recurrent disease at the site of the primary tumor, 8 had regionally recurrent disease, and 1 had both. Regional tumor recurrences included lymph-node metastases, subcutaneous metastases in the neck after prior lymph-node dissection, and skin metastases. In total 22 tumor recurrences were treated in 19 patients. Median time between diagnosing the current tumor recurrence and starting PEG-IL-2 treatment was 1 month (range 0.5-14 months). In the 2 patients with the largest delay (6 and 14 months, in patient no. 13 and 10, respectively) the current tumor recurrence was treated with palliative laser debulking and/or chemotherapy with methotrexate, before PEG-IL-2 was given.

Treatment duration is shown in Table 2. More than 90% of the PEG-IL-2 injections were given in the out-patient clinic. In local tumor recurrences, preferably the vital borders, and not necrotic central parts were injected. In the larger tumor masses, variable sites were injected. Depending on the fragility of the tumor, leakage of fluid was sometimes observed. This appeared problematic in patient no. 15 only. For treatment of lymph-node metastases in patients nos. 2, 3, 8, 12, and 14, injection site and depth were determined, and controlled regularly, by ultrasound.

Table 2. *HNSCC locoregional recurrences and intratumoral PEG-IL-2 treatment.*

Pat. no.	Treated lesions		Total weeks of treatment	Response (duration, weeks)	Patient survival (weeks)	Locoregional side effects	Eosinophils, max. level[c] ($\times 10^6$/l)
	Site[a]	Size (cm^2)[b]					
1	subc.	7.2	8	CR (91)	102+	swelling, redness	1140
2	l.n. (2)	3.1; 3.2	2	Early Progression	9	swelling, redness	962
3	l.n.	4.3	8	SD (40)	59	swelling, redness, itching	2436
4	subc.	10.8	4	PD	20	none	320
5	prim.	3.8	6	PD	23	swelling, redness	720
6	prim.	12.0	6	PD	24	enhanced tumor necrosis	231
7	prim.	1.5	8	SD (8)	39	swelling, redness	726
8	l.n.+prim.	5.5; 5.3	8	SD (8)	22	swelling, redness, mild pain	710
9	prim.	36.0	2	Early Progression	14	none	97
10	prim.	42.0	4	PD	8	none	186
11	prim.	4.9	4	PD	8	none	162
12	l.n.	1.6	4	SD (57+)	57+	swelling, redness, itching	3515
13	prim.	not measurable	14	not measurable	31	swelling, redness	688
14	l.n.	20.0	8	SD (23)	52+	swelling, redness, itching	4653
15	prim.	6.3	4	PD	38+	none	70
16	l.n.	23.4	8	SD (12)	36+	mild pain	99
17	prim.	15.0	4	PD	11	none	152
18	skin (2)	3.6; 2.0	2	not evaluable	2	swelling, redness	n.m.
19	prim.	6.0	4	PD	6	mild pain	260

[a] subc., subcutaneous; l.n., lymph node; prim., primary tumor site.

[b] Product of largest biperpendicular diameters.

[c] Normal count $<400\times10^6$/l. n.m., not measured.

Response

Tumor response and patient survival are shown in Table 2. Seventeen patients were evaluable for response. One CR (1/17, 6%) was obtained in a 69-year old man with a Karnofsky score of 80%, who previously had radiotherapy, laryngectomy, and bilateral lymph-node dissection for poorly differentiated SCC of the larynx (patient no. 1). He was treated with PEG-IL-2 for a nodular subcutaneous metastasis in the left neck. Shrinkage of this lesion was first observed after 3 weeks of treatment. After 8 weeks a flat superficial skin lesion remained, which could not be injected anymore. Treatment was stopped then, and 4 weeks later the lesion had disappeared completely (Fig. 1). The CR persisted for 91

veeks. Then a tumor recurrence occurred in the pharynx, which was not accessible to njection. Radiotherapy was started.

n 6 patients SD was recorded (duration 8-57* weeks). Five of them received 8 weeks of reatment, according to the protocol. One patient (no. 12) refused further treatment after l weeks, because of emotional stress. The 3 best SDs, lasting 57*, 40, and 23 weeks, were obtained in patients with single lymph-node metastases (patients nos. 12, 3, and 14, espectively). In patient no. 3, treated for a lymph-node metastasis of a larynx carcinoma, SD was accompanied with loss of contrast enhancement of the lesion on CT scan, and absence of neoplastic cells in an ultrasound-guided cytological puncture at the end of PEG-IL-2 treatment.

Patients with progressive tumor growth at evaluation after 4 weeks, or before completing he next 4 weeks of treatment were recorded as PD. In patient no. 6, who was treated for ocally recurrent carcinoma of the oropharynx, an impressive enhancement of central umor necrosis occurred, with expectoration of an huge necrotic mass 6 weeks after topping treatment. Nevertheless, the outer tumor margins gradually increased. Ultrasound xamination suggested progressive central tumor necrosis in 1 out of the 5 patients whose ymph-node metastases were evaluated that way (patient no. 8).

Two patients were not evaluable for response. Patient no. 13 had unmeasurable disease. Because of subjective improvement, PEG-IL-2 treatment was continued till 14 weeks. Patient no. 18, a 66-year old man, unexpectedly died during his sleep, about 36 hours after the 5th PEG-IL-2 administration for 2 skin metastases on the cheek. Autopsy was not allowed. The cause of death remained uncertain.

n 5 patients (nos. 3,4,14,16, and 17) chemotherapy was given for progressive tumor growth during or after PEG-IL-2 therapy. This did not result in objective responses. Median survival from the start of PEG-IL-2 treatment was 23 weeks for the total group, and also for the 17 evaluable patients, and >45 weeks for the 6 patients who achieved SD.

Toxicity

All patients were evaluable for toxicity. Treatment was well tolerated. Mild pain following njections was experienced by 3 patients. Except for low-grade fever in 1 patient (no. 19), no systemic toxicity was observed. Renal and hepatic function remained unchanged. In 1 patient (no. 8) treatment was interrupted 1 week because of an intercurrent respiratory nfection, and continued afterwards without problems.

During treatment, a locoregional reaction with swelling and redness of the neck and/or face on the side of injection developed in 10 patients (Table 2). Three of these patients complained of locoregional itching as well. The swelling consisted of thickening of the

skin and subcutaneous edema, as was confirmed by ultrasound examination. This reaction generally started after 1 week of treatment, and decreased gradually from 1-2 weeks after stopping treatment. These locoregional side effects were most impressive in patients who received PEG-IL-2 into lymph-node metastases.
Also because of this mild toxicity in general, a causative relation between PEG-IL-2 treatment and the sudden unexpected death in patient no. 18, as described above, was felt unlikely.

Hematological effects

Hematological blood counts revealed an increase in the number of eosinophils during treatment in 9 patients. Remarkably, these were the same patients who also developed regional swelling and redness during treatment (Table 2). In most patients (7 out of 9) the highest levels of eosinophils were observed after 2 weeks of PEG-IL-2 treatment. They returned to normal after stopping treatment. No consistent changes occurred in peripheral blood lymphocyte counts (data not shown).

Anti-IL-2 antibodies

In none of the 16 patients tested, anti-IL-2 serum antibodies could be detected.

Immunohistochemical analysis

Histopathological results are represented in Table 3. Pretreatment tumor samples included biopsies from the current tumor lesion (n=9), and biopsies from previously treated tumor recurrences (n=5) or primary tumors (n=5). The latter 10 concerned patients in whom malignancy of the PEG-IL-2-treated lesion was confirmed cytologically. Most tumors were moderately differentiated squamous-cell carcinomas. A variable intensity of the mononuclear cell infiltrate was observed. HLA class I-expression by 25% of the tumor cells or less, was observed in 9 out of 18 in the respect evaluated pretreatment tumor samples, while HLA class II-expression was absent (≤5% tumor cells stained) in 15 pretreatment samples. Tumor biopsies after 4 weeks of PEG-IL-2 treatment were obtained in 3 patients with local tumor recurrences (nos. 5,6,7), who had either PD or short-term SD. The intensity of the mononuclear cell infiltrate before PEG-IL-2 treatment happened to be low in all 3. No differences were observed after PEG-IL-2 injections, neither with regard to the infiltrating cells, nor to HLA expression by tumor cells. In patient no. 10, enhanced HLA class II expression by tumor cells was observed in a tumor sample obtained at autopsy.

Table 3. *Histopathological evaluation and HLA antigen expression on tumor lesions from patients treated with intratumoral PEG-IL-2 injections because of locoregionally recurrent HNSCC*

Patient no.[a]	Evaluated tumor lesion[b]	Histopathol. grade[c]	Intensity of mononuclear cell infiltrate[d]	HLA antigen expression by tumor cells[e]	
				class I	class II
1	previous recurrence	poorly	++	≤5%	≤5%
2	primary tumor	moderately	+++	76-100%	51-75%
3	primary tumor	moderately	++	6-25%	≤5%
4	primary tumor	moderately	++	51-75%	6-25%
5	before PEG-IL-2	moderately	-	51-75%	26-50%
	after PEG-IL-2	moderately	-	51-75%	26-50%
6	before PEG-IL-2	moderately	+	51-75%	≤5%
	after PEG-IL-2	moderately	n.i.	51-75%	≤5%
7	before PEG-IL-2	well	+	6-25%	≤5%
	after PEG-IL-2	well	+	6-25%	≤5%
8	previous recurrence	moderately	++	51-75%	≤5%
9	previous recurrence	poorly	-	≤5%	≤5%
10	previous recurrence	moderately	-	6-25%	≤5%
	autopsy	moderately	+	26-50%	26-50%
11	previous recurrence	well	+	76-100%	≤5%
12	primary tumor	moderately	++	6-25%	≤5%
13	before PEG-IL-2	moderately	+	6-25%	≤5%
14	primary tumor	poorly	+	6-25%	≤5%
15	before PEG-IL-2	well	-	n.d.	n.d.
16	before PEG-IL-2	well	+	26-50%	≤5%
17	before PEG-IL-2	moderately	+	26-50%	≤5%
18	before PEG-IL-2	poorly	+	≤5%	≤5%
19	before PEG-IL-2	moderately	++	51-75%	≤5%

[a] Numbers corresponding to Table 2.

[b] Pretreatment tumor samples included biopsies from the current tumor recurrence (before PEG-IL-2), from the primary tumor, or from a previous tumor recurrence which was treated with surgery or radiotherapy. Posttreatment tumor samples were obtained immediately after 4 weeks of PEG-IL-2 treatment, or at autopsy.

[c] Grade of squamous cell carcinoma.

[d] -, no or only very few mononuclear cells; +, occasional patch; ++, patchy rim; +++, continuous rim of infiltrating cells; n.i., not interpretable due to absence of tissue surrounding the tumor in the specimen.

[e] Immunohistochemically detected with anti-HLA class I MAb HC10, or anti-HLA-DR rabbit serum on paraffin sections. Expression of antigens by the tumor cells was estimated as a percentage of the total number of tumor cells in the slide, and scored in 1 out of 5 categories. n.d., not done.

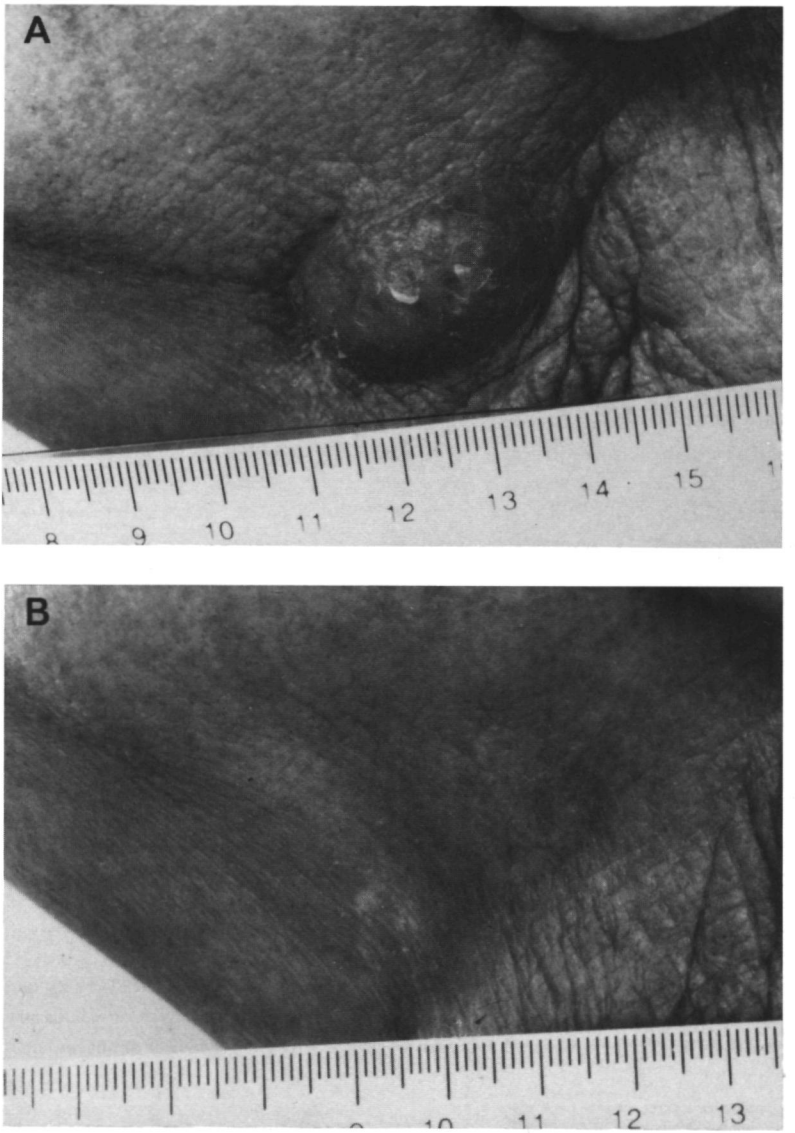

Figure 1. Nodular subcutaneous tumor recurrence (30×24 mm, cytologically confirmed squamous-cell carcinoma, with documented progressive growth) in the upper jugular region of the left neck, in a 69-year old man who was previously treated with radiotherapy, laryngectomy, and bilateral neck lymph-node dissection for poorly differentiated squamous-cell carcinoma of the larynx. *a*, before PEG-IL-2 treatment. *b*, complete response, 4 weeks after stopping treatment with 3 times weekly 200,000 U PEG-IL-2 injections for 8 weeks.

Discussion

From this study we can conclude that intratumoral injection of PEG-IL-2, 3 times weekly at intermediate doses, in patients with HNSCC is feasible. Treatment was applicable in the out-patient clinic, and toxicity was mild. The response rate obtained in a selected group of patients with locoregionally recurrent disease was low, as 1 objective response was recorded in 17 evaluable patients (6%). Nevertheless, the longlasting CR, in a patient for whom no other treatment was available anymore, indicates that a highly effective antitumor mechanism can be induced in certain circumstances. In addition, 3 patients achieved SD of substantial duration.

Considering the present and previous studies [13,14,18] on locoregional IL-2-based therapy in HNSCC, a tumor response in patients with large or very fast-growing tumors appears unlikely. While it has been suggested that responses occurred at the primary tumor site only [13,18], in our study, the patient with the CR and the 3 with the best SDs all had a single regional tumor recurrence, of relatively small size. These 4 patients developed locoregional edema, and induration and redness of the skin, as well as 6 other patients. Temporary regional edema has also been described during local IL-2 therapy in HNSCC patients [11,16,18]. This local reaction might be related to increased microvascular permeability at the site of IL-2 application, as was demonstrated in an experimental situation [27]. After subcutaneous or intradermal IL-2 [28] or PEG-IL-2 [24] injection in humans a local indurative and erythematous reaction, resembling a delayed-type hypersensitivity (DTH) reaction, was described to consist of mononuclear cell infiltration, and to increase with the dose of PEG-IL-2 [24]. It is tempting to say that this local inflammatory reaction might contribute to the antitumor effect of intratumoral PEG-IL-2 therapy.

Systemic eosinophilia is well described in cancer patients treated with systemic IL-2 therapy, and has been shown to be mediated by IL-5, produced by IL-2-activated T cells [29]. Remarkably, in the present study eosinophilia occurred in the patients who developed therapy-induced locoregional swelling only, while the highest levels were found in the patient with the CR, and the 3 best SDs. An association between eosinophilia and a favorable tumor response was found before by Atzpodien *et al.* [28] in cancer patients receiving subcutaneous IL-2 and IFNα therapy. In the line-10 guinea-pig tumor, in which we evaluated intratumoral PEG-IL-2 therapy before [19], an intense inflammatory reaction with eosinophils, and also T lymphocytes, macrophages and fibroblasts was observed at the site of regressing tumors [30]. This suggests that eosinophils play a role in the PEG-IL-2-induced antitumor effect. Evidence for eosinophil-mediated tumor-cell killing *in vivo* was

provided by Tepper *et al.* [31], who reported IL-4-induced antitumor effects to be dependent on local eosinophil infiltration. Independent of immunotherapy, the grade of tumor-infiltrating eosinophilia was found to be a prognostic marker in HNSCC [32]. In the present study, no sufficient material could be obtained for histopathological analysis of PEG-IL-2-induced antitumor reactions. Posttreatment biopsies from local tumor recurrences in 4 non-responding patients did not show infiltration of eosinophils, nor other histopathological changes. For thorough histological and immunohistochemical analysis of PEG-IL-2-induced locoregional reactions, possibly contributing to the antitumor effect, an alternative study design, with intratumoral injections prior to tumor resection and regional lymph-node dissection, appears more appropriate.

Cytotoxic $CD8^+$ T cells recognize antigens when presented together with HLA class I antigens. We evaluated the level of HLA class I antigen expression by the HNSCCs in this study with immunohistochemical methods. A previous tumor recurrence of the patient with the CR (Table 3, no. 1) and the primary tumors of the 3 patients with the best SDs (Table 3, nos. 3,12, and 14) all had a low level (≤25%) of HLA class I expression. Since we are not informed about the HLA class I expression of the PEG-IL-2-treated lesions in these patients, nor about any therapy-induced changes in HLA class I expression, no certain conclusions are allowed. Remarkably, however, the PEG-IL-2-responsive line-10 guinea-pig tumor immunohistochemically also showed low expression of major histocompatibility (MHC) class I antigens [30]. These findings do not support a major role for $CD8^+$ T cell-mediated cytotoxicity in the antitumor effect induced by intratumoral PEG-IL-2 injections.

Sacchi *et al.* [33] found that the growth of HNSCC xenografts in nude mice with eliminated NK activity was inhibited by local IL-2 administration, and suggested that this was a direct effect of IL-2 itself. This was supported by the demonstration of functional intermediate-affinity and high-affinity IL-2 receptors on some HNSCC cell lines [34]. Moreover, growth of these tumor-cell lines, and not normal keratinocytes, was inhibited by IL-2 *in vitro* [34]. Fresh tumor samples, necessary for immunohistochemical analysis of IL-2 receptor expression, were not available in the present study. However, this possible mechanism for IL-2-induced tumor regression in HNSCC certainly deserves further attention in prospective clinical studies.

In rabbits and mice, PEG-IL-2 was demonstrated to be less immunogenic than IL-2 [35]. We did not find anti-IL-2-antibody production following intratumoral PEG-IL-2 administration in the HNSCC patients within this study. The relevance of this finding is uncertain.

In the present study, a single, intermediate dose-level for PEG-IL-2 was chosen. This dose

was based on our experimental results with intratumoral PEG-IL-2 in the fast-growing line-10 tumor, where a dose-response relationship was found for low to intermediate dose levels. It is uncertain whether the present dose is optimal for HNSCC. In previous studies with locoregional IL-2 administration in HNSCC, however, no indications were found for high IL-2 doses to give better results [15,16]. In contrast, most objective clinical responses were obtained especially with daily locoregional administration of relatively low doses (200-30,000 U) of IL-2 [13,15,16].

In conclusion, a minority of patients with locoregionally recurrent HNSCC appears to benefit from intratumoral PEG-IL-2 injections. Therapy is well tolerated. The antitumor mechanism is unknown. Both a locoregionally induced inflammatory-like reaction, eosinophils, and a direct effect via binding to IL-2 receptors on HNSCC tumor cells might be involved. Increased knowledge on the underlying mechanism would enable identification of patients susceptible to this kind of locoregional immunotherapy.

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Chapter 8

Biodistribution of interleukin-2 and PEG-interleukin-2 in the guinea-pig following intratumoral injection

Submitted for publication as:

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Summary

Background: Interleukin-2 (IL-2) is rapidly cleared from the circulation. In several experimental studies on immunotherapy with IL-2 local administration was found a prerequisite for the induction of an effective host antitumor immune response. In the line-10 guinea-pig tumor enhanced efficacy was demonstrated when polyethylene glycol (PEG)-modified IL-2 was used. We examined whether the difference in efficacy of intratumorally injected IL-2 and PEG-IL-2 was related to the duration of retention at the site of the tumor and/or the tumor-draining lymph nodes.

Methods: IL-2 and PEG-IL-2 were radioiodinated with ^{125}I and ^{131}I, respectively. A mixture of both compounds was injected intratumorally into line-10 guinea-pig tumors. At several intervals groups of 4 animals were killed. Selective organs were removed and radioactivity was measured.

Results: A gradual decrease of activity in the tumor was observed for both ^{125}I-IL-2 and ^{131}I-PEG-IL-2 following intratumoral injection. Uptake of both labeled compounds was found in the tumor-draining lymph node. Blood levels for ^{131}I-PEG-IL-2 were significantly higher than for ^{125}I-IL-2 ($p<0.007$) from 3 h up to 48 h after intratumoral injection.

Conclusion: No evidence was found for prolonged retention of PEG-IL-2 as compared to IL-2 in the tumor or the regional lymph node to explain the enhanced efficacy of intratumoral PEG-IL-2 administration. The higher blood levels for ^{131}I-PEG-IL-2 suggest that circulatory activity might contribute to the antitumor effect of intratumoral IL-2-based therapy.

Introduction

The 15-Kd protein interleukin-2 (IL-2) is a product of T lymphocytes which plays an essential role in the generation of the immune response and the clonal expansion of lymphocytes [1]. Endogenous IL-2 mediates its effect locally in a paracrine and/or autocrine fashion by binding to IL-2-specific cell-surface receptors. Since several years recombinant IL-2 has been studied as an immune-stimulant in anticancer therapy [2]. IL-2 is rapidly cleared from the circulation. After i.v. bolus injection in humans a high serum peak-level is obtained, decreasing initially with a half-life of 6-13 min ($t\alpha$), followed by a slower elimination phase with a half-life of 70-85 min ($t\beta$) [3,4]. Subcutaneous (s.c.) injection and 30-min intraperitoneal (i.p.) infusion result in much lower but fairly constant serum levels for about 8 h [4]. Renal filtration appears the major route of clearance [4]. Because of its rapid clearance IL-2 is mostly administered by repeated high-dose i.v. bolus [5], continuous infusion [6] or s.c. injections [7] in order to obtain lasting serum levels at an assumed therapeutic level. With all schedules mentioned objective responses were obtained in selected patient groups. However, which serum levels are most beneficial is not clear [8]. Moreover, it is not known where IL-2 generates its antitumor effect, *i.e.* in the circulation, at the site of the tumor, or in some specific organ. After i.v. bolus injection of radioiodinated IL-2, specific accumulation has been demonstrated in the liver and to a lesser extent in the spleen [9,10]. After i.p. administration, on the other hand, no organ-specific uptake was found [9]. Whether there is uptake of IL-2 into tumors following systemic administration is not known.

We previously studied the antitumor effects of locoregionally injected IL-2 and polyethylene glycol-modified IL-2 (PEG-IL-2) in the line-10 guinea-pig tumor, and found PEG-IL-2 to be superior to IL-2, at less frequent administration [11]. The intratumoral administration route was obligatory for the obtained effect [11]. PEG-IL-2 has enhanced solubility and a 10- to 20-fold prolonged circulatory half-life as compared to IL-2 [12]. The latter is supposed to explain the higher antitumor activity of PEG-IL-2 compared to IL-2 after i.v. administration [12,13]. We hypothesized that prolonged locoregional retention of PEG-IL-2 might be responsible for the enhanced antitumor activity as compared to IL-2 after intratumoral injection. In the present study we investigated the distribution over time of radioiodinated IL-2 and PEG-IL-2 in guinea-pigs after intratumoral injection, with special emphasis on the tumor and the tumor-draining lymph nodes. Knowledge on this distribution might contribute to the elucidation of the working mechanism of intratumoral IL-2 therapy in general, and might be relevant for application of experimental data in the human situation.

Materials and methods

Animal tumor model

Sewall-Wright inbred strain-2 guinea-pigs were obtained from the National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD, and used when 2 to 3 months old, weighing about 500 g. Line-10 hepatocellular carcinoma cells, syngeneic to strain-2 guinea-pigs, were maintained as frozen stock, and propagated intraperitoneally. Tumor cells were used in their 12th passage. Intradermal inoculation of 1×10^6 tumor cells in the anterior left flank was performed on day 0. In previous experiments this led to progressive local tumor growth and development of regional axillary lymph node metastases within one week [11].

Radioiodination of IL-2 and PEG-IL-2

Recombinant human IL-2 (Proleukin, Eurocetus, Amsterdam, The Netherlands; specific activity 18.0×10^6 U/mg) was radiolabeled with ^{125}I using the glucoseoxidase-lactoperoxidase method, essentially according to the manufacturer's instructions. Briefly, 1 mg lyophilized IL-2 was reconstituted in 250 µl distilled water. IL-2 (350 µg) was incubated with 50 µl enzymobead suspension (Biorad, Richmond CA) and 7.0 mCi $Na^{125}I$ (Amersham International, Amersham, UK) in 0.15 M phosphate buffer, pH 7.4. The reaction was initiated by adding D(+)glucose to the final concentration of 4 mg/ml. After 30 min the enzymobeads were spun down (2,000×g, 5 min), and the supernatant was eluted with phosphate buffered saline on a Sephadex G-25 column (PD-10; Pharmacia, Uppsala, Sweden) to separate the labeled IL-2 from the free ^{125}I. The void fractions containing 6% of the radioactivity, were pooled and sterilized through a 0.2 µm filter.

Recombinant human IL-2 modified by the covalent attachment of 2-3 PEG M, 7,000 molecules per IL-2 molecule (PEG-IL-2; specific activity 5.5×10^6 U/mg IL-2 protein) was provided by Eurocetus, Amsterdam, The Netherlands. PEG-IL-2 was labeled with ^{131}I according to the enzymobead method as described above. PEG-IL-2 (5.5×10^6 U) was incubated with 50 µl enzymobead suspension and 1.8 mCi $Na^{131}I$ (Medgenix, Fleurus, Belgium) in 0.15 M phosphate buffer, pH 7.4. The reaction was initiated as described above. Gelfiltration indicated a labeling efficiency of 85%.

Experimental design

Twenty-eight guinea-pigs with 10-day old palpable line-10 tumors on the flank (diameter 10-12 mm) were divided into 7 equal groups. Animals received an intratumoral injection with 0.2 ml of the injection mixture, containing 14 µCi ^{125}I-IL-2 (200,000 U) and 28 µCi

^{131}I-PEG-IL-2 (100,000 U). At 5 min and 1, 3, 6, 12, 24, and 48 h after injection groups of 4 animals were given an intracardial injection of pentobarbital (60 mg) and were bled. Selective tissues, including the tumor and the first tumor-draining axillary lymph node, were dissected, weighed and counted in a well-type gamma counter (Pharmacia-LKB, Sweden) along with injection standards to correct for physical decay. Data were represented as percent of the injected dose per dissected tissue (% ID; for tumors and thyroid glands), or per gram organ weight (%ID/g) for ^{125}I-IL-2 and ^{131}I-PEG-IL-2 separately.

Statistical analysis

For each time point mean values ± SD were calculated for the group of 4 animals, separate for ^{125}I-IL-2 and ^{131}I-PEG-IL-2. After log-transformation Students' *t*-test for paired differences was used for comparison between values of ^{125}I-IL-2 and ^{131}I-PEG-IL-2 at each time point. This was done separately for tumor, lymph node and blood. The Bonferroni-correction was used for testing on each of the multiple time points. A difference was called statistically significant if the resulting *p*-value of the *t*-test is <0.007 (<0.05/7).

Results

The intratumoral activity of ^{125}I-IL-2 and ^{131}I-PEG-IL-2 over time is represented in Fig. 1. At 5 min after injection about half of the injected dose of both labeled compounds was found in the tumor. The intratumoral levels of both compounds decreased rapidly to about 10% of the injected doses at 3 h, and then more slowly to about 3% at 24 h after injection. Significant (p<0.007) higher values for ^{131}I-PEG-IL-2 were found at 1h and 3h after injection. At 24h and 48h the values for ^{125}I-IL-2 were significantly higher, although absolute intratumoral levels were very low for both compounds then.

Biodistribution data for intratumorally injected ^{125}I-IL-2 and ^{131}I-PEG-IL-2 are shown in Table 1. Uptake of both labeled compounds in the tumor-draining lymph node (indicated as % ID/gram organ weight) was clearly higher than in any other organ studied. Although the uptake of ^{131}I-PEG-IL-2 in the tumor-draining lymph node appeared higher than that of ^{125}I-IL-2, no significant differences were found. The uptake values of both labeled compounds for liver and spleen were very low, as were those for lung, intestine and muscle (data not shown).

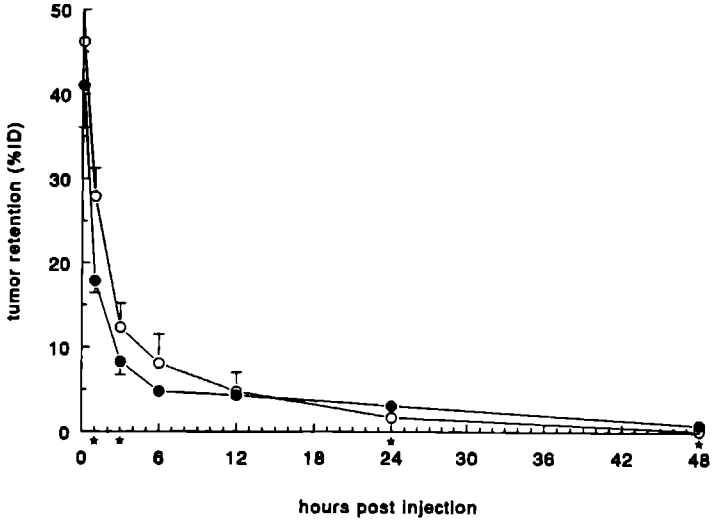

Figure 1. Intratumoral levels (in % ID/tumor) of ^{125}I-IL-2 (●) and ^{131}I-PEG-IL-2 (○) following simultaneous intratumoral injection into 10-day old intradermal line-10 guinea-pig tumors. Points, mean values of 4 animals; bars, SD. Time-points at which statistically significant differences were obtained ($p<0.007$) are indicated with *.

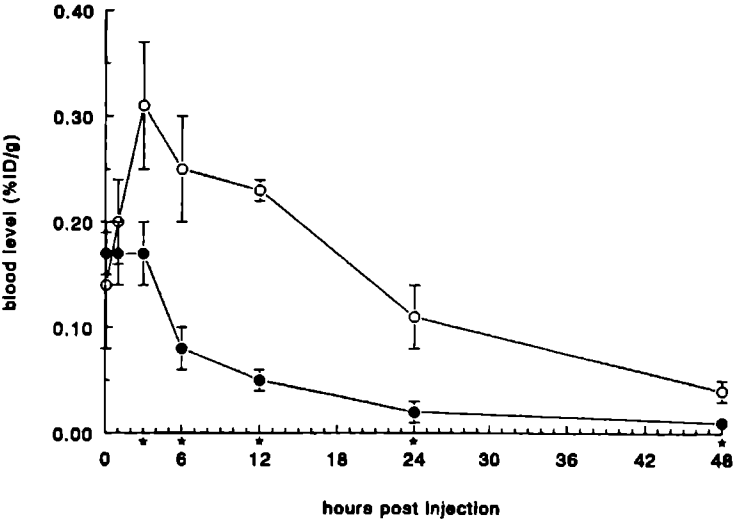

Figure 2. Blood levels (in % ID/g blood) of ^{125}I-IL-2 (●) and ^{131}I-PEG-IL-2 (○) following simultaneous intratumoral injection into 10-day old intradermal line-10 guinea-pig tumors. Points, mean values of 4 animals; bars, SD. Time-points at which statistically significant differences were obtained ($p<0.007$) are indicated with *.

Table 1. *Biodistribution of ^{125}I-IL-2 (A) and ^{131}I-PEG-IL-2 (B) in guinea-pigs following mixed intratumoral injection.*

	% ID/organ; mean of 4 animals ± SD Time post injection						
	5 min	1 h	3 h	6 h	12 h	24 h	48 h
Tumor							
A	41.03±4.96	17.88±1.40	8.29±1.55	4.80±0.56	4.30±0.19	3.06±0.66	0.85±0.43
B	46.20±6.37	27.89±3.36	12.33±2.89	8.11±3.42	4.75±2.24	0.69±0.48	0.15±0.06
Thyroid							
A	0.07±0.03	0.36±0.05	1.07±0.21	1.30±0.29	1.74±0.92	3.21±1.48	3.19±0.74
B	0.02±0.01	0.07±0.01	0.27±0.06	0.47±0.12	0.91±0.48	2.27±1.05	2.81±0.72
	% ID/g organ weight; mean of 4 animals ± SD Time post injection						
	5 min	1 h	3 h	6h	12 h	24 h	48 h
Lymph node							
A	10.37±4.01	4.57±0.73	1.54±1.17	0.87±0.40	0.45±0.30	0.20±0.19	0.14±0.08
B	10.66±3.42	6.34±1.19	4.06±2.57	2.40±0.52	0.91±0.36	0.21±0.02	0.04±0.01
Blood							
A	0.17±0.02	0.17±0.03	0.17±0.03	0.08±0.01	0.06±0.01	0.03±0.01	0.01±0
B	0.14±0.06	0.20±0.04	0.31±0.06	0.25±0.05	0.23±0.01	0.11±0.03	0.04±0.01
Liver							
A	0.09±0.01	0.09±0.02	0.09±0.02	0.04±0.01	0.03±0.01	0.01±0.01	0.01±0.01
B	0.05±0.01	0.06±0.03	0.11±0.03	0.07±0.02	0.06±0.01	0.03±0.01	0.01±0.01
Spleen							
A	0.09±0.02	0.12±0.02	0.08±0.02	0.04±0.01	0.03±0.01	0.01±0.01	0.01±0.01
B	0.03±0.01	0.05±0.01	0.09±0.04	0.06±0.02	0.07±0.01	0.03±0.01	0.01±0.01
Kidney							
A	0.49±0.18	1.24±0.16	0.53±0.13	0.16±0.03	0.08±0.01	0.03±0.01	0.01±0.01
B	0.14±0.07	0.43±0.08	0.72±0.08	0.56±0.06	0.41±0.06	0.14±0.04	0.04±0.01

A mixture of ^{125}I-IL-2 and ^{131}I-PEG-IL-2 was injected intratumorally into 10-day old intradermally growing line-10 guinea-pig tumors. At the indicated time-points following injection animals were killed and tissues were obtained for measurement of radioactivity.

Blood levels are shown in Fig. 2. From 3 h after injection, when ^{131}I-PEG-IL-2 reached a peak blood level, till the end of the experiment blood levels of ^{131}I-PEG-IL-2 were significantly higher ($p<0.007$) than the levels of ^{125}I-IL-2.
The experiment, including the labeling procedure, was performed twice. Essentially the same results were obtained (data not shown).

Discussion

In several experimental tumor models evidence was obtained that repeated local, and not systemic, administration of IL-2 could elicit an effective antitumor immune response [14-17]. It was suggested that locally supplied IL-2 directly activated tumor-sensitized immune cells at the tumor site, thus bypassing an otherwise failing helper T cell function [18]. We previously described that guinea-pigs with palpable line-10 tumors on the flank and regional lymph-node micrometastases could be cured, and developed systemic anti-line-10 immunity, by 3-times weekly intratumoral PEG-IL-2 injections, whereas IL-2 caused tumor-growth inhibition only [11]. The obligatory intratumoral injection route [11] suggested that the antitumor effect of PEG-IL-2 was generated at the tumor site. We hypothesized that intratumoral injection of PEG-IL-2 resulted in more prolonged retention at the site of the tumor and/or the tumor-draining lymph-nodes than IL-2. This was supported by the observation in humans that PEG-IL-2 following intradermal or subcutaneous injection showed a greater local indurative and erythematous reaction with longer duration than IL-2 [19]. The results of the present study, however, do not confirm our hypothesis. Although from 1 to 3 h after injection higher tumor values were found for ^{131}I-PEG-IL-2, protracted retention in the tumor, and uptake into the tumor-draining lymph node following intratumoral injection were comparable for PEG-IL-2 and IL-2. The unexpected sustained locoregional levels of ^{125}I-IL-2 might be partly explained by limited denaturation of ^{125}I-IL-2, since analysis of the ^{125}I-IL-2 preparation by sodium-dodecyl sulphate (SDS) polyacrylamide gelelectrophoresis indicated that 90% migrated as a 15-Kd compound, while 10% migrated as a high molecular weight compound (>100 Kd; data not shown).
Uptake of both labeled compounds in liver and spleen was very low, and probably of no relevance. Kidney values were somewhat higher, which is concordant with the renal excretion mechanism. Thyroid uptake (in % ID/thyroid) represents dehalogenated ^{125}I and ^{131}I.
A clear difference in biodistribution of ^{125}I-IL-2 and ^{131}I-PEG-IL-2 was observed at the

circulatory level. Intratumoral administration proved to result in significantly higher blood levels for ^{131}I-PEG-IL-2 than for ^{125}I-IL-2. This probably is related to the higher circulatory half-life of PEG-IL-2 as compared to IL-2 [12]. Only radioactivity, and no bioactivity was measured in this study. Yet, it should be considered that the circulatory levels following intratumoral PEG-IL-2 injection in the present model might have contributed to the obtained antitumor effect by activation of host immune mechanisms [11]. The experiments of Forni *et al.* [14,20] also suggested that local IL-2 immunotherapy encompassed more than a purely local reaction. They found that the antitumor response induced by peritumoral IL-2 injections in mice challenged with tumor cells admixed with lymphocytes was abrogated in immunosuppressed animals. The circulatory PEG-IL-2 levels in the guinea-pigs might be high enough to activate intermediate-affinity IL-2 receptors from natural killer (NK) cells. Other investigators found evidence that these cells are involved in the antitumor response induced by local IL-2 supply as well [18,20].

If circulatory levels of (PEG-)IL-2 are relevant for the effect of intratumoral (PEG)-IL-2 therapy, this has important consequences for application of the experimental data in humans, since equal intratumoral doses in guinea-pigs and man might provide comparable levels at the injection site, but much lower circulatory levels in humans.

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Chapter 9

Summary and general discussion

Interleukin-2 (IL-2) is one of the most extensively studied immune-stimulants in anticancer immunotherapy. Natural IL-2 has a central role in the generation of the immune response, and enhances proliferation and activity of both specific and non-specific immune-competent cells. In therapeutical studies generally high-dose systemic administration is used. This way, remarkable tumor responses have been obtained in a small percentage of cancer patients, by a still incompletely understood working mechanism. Considering the biological and pharmacological characteristics of IL-2, and the potential antitumor effects of tumor-infiltrating lymphocytes, however, locoregional administration, *i.e.* at the site of the tumor, might be preferable. Moreover, in several experimental tumor models enhanced efficacy of IL-2 after locoregional application, including the generation of a specific antitumor immune response, has been demonstrated. The background and experimental data which formed the basis for further exploring the value of locoregional immunotherapy with IL-2 are described in detail in chapter 2.

Head and neck squamous-cell carcinoma (HNSCC) appeared suitable to locoregional IL-2 administration because of its predominantly locoregional tumor spread, accessibility to injection, and localization close to extensive submucosal and nodal lymphatic tissue. We studied the effects of 10 daily low-dose IL-2 injections in patients with locoregionally far-advanced non-pretreated HNSCC (chapter 4). Injections were given perilymphatically, *i.e.* around the tumor-draining lymph nodes, as was described previously in HNSCC by Cortesina *et al.* (Cancer 1988). In contrast to them we did not observe tumor responses in 15 patients treated. Furthermore, histopathological evaluation of pre- and post-treatment tumor biopsies from 10 patients revealed no treatment-induced changes. The absence of any effect might be related to the large tumor masses in our patient group.

To obtain more information on the optimal dose and route for locoregionally administered IL-2 in established malignant disease, we performed a series of experiments in the line-10 guinea-pig tumor (chapter 5). Next to IL-2, polyethylene-glycol modified IL-2 (PEG-IL-2) was used. PEG-IL-2 had enhanced solubility and prolonged circulatory half-life, eventually permitting less frequent administration. Data on locoregional application of this formulation were not available so far. We found that guinea-pigs with palpable tumors on the flank and micrometastases in the regional lymph nodes could be cured by repeated intratumoral injections with PEG-IL-2. Moreover, these animals had developed systemic anti-line-10 immunity, protecting them against line-10 tumor rechallenges on the other flank. PEG-IL-2 was superior to IL-2 since higher efficacy was obtained at less frequent administration. The intratumoral administration route was obligatory for the antitumor

effect. This was in concordance with previous experimental results from other investigators with IL-2, and strongly suggested that the antitumor effect was generated at the site of the tumor.

Subsequently, the in the experimental setting optimal PEG-IL-2 schedule was applied in HNSCC patients to study the feasibility and the antitumor efficacy (chapter 7). Nineteen patients with locoregionally recurrent HNSCC, for whom no curative treatment was available, were treated with 3-times weekly intratumoral injections with 200,000 IU of PEG-IL-2, in courses of 4 weeks. Systemic toxicity, well known from high-dose systemic IL-2 therapy, did not occur. Within 17 evaluable patients, 1 complete response (CR; duration 91 weeks) and 6 stable diseases (SD; duration 8-57+ weeks) were observed. The CR and the 3 best SDs occurred in patients with a single regional tumor recurrence of relatively small size. These 4 patients, besides 4 others, developed temporary regional swelling and redness, and high levels of circulating eosinophils.

Knowledge on the working mechanism of intratumoral PEG-IL-2 immunotherapy might contribute to optimizing treatment schedules and identification of patients susceptible to this kind of treatment. In the line-10 guinea-pig tumor histological and immunohistochemical analysis revealed that PEG-IL-2-induced tumor regression was accompanied by in intense peritumoral inflammatory reaction of strikingly mixed composition (chapter 6). Involvement of T lymphocytes in the induced antitumor effect was suggested by the infiltration of $CD8^+$-like T lymphocytes into the substance of the tumor. Furthermore, the PEG-IL-2-induced antitumor effect was completely abrogated by pretreatment of the animals with antithymocyte serum, and the obtained anti-line-10 immunity could be transferred to naive animals by spleen lymphocytes (Balemans *et al,* Cancer Immunol Immunother 1993). Whether the temporary locoregional swelling and redness during PEG-IL-2 treatment in 8 HNSCC patients was caused by a similar inflammatory reaction remains speculative, since it appeared very difficult to obtain sufficient material for histological and immunohistochemical analysis in these patients (chapter 7). In chapter 6 the possible role for other infiltrating cells observed in PEG-IL-2-treated line-10 tumors is discussed. Especially eosinophils might be important since these cells were observed in high numbers at the site of PEG-IL-2-treated guinea-pig tumors (chapter 6), and also at high circulatory levels in the HNSCC patients with the CR and the 3 best SDs after PEG-IL-2 therapy (chapter 7).

Because T lymphocytes are MHC-restricted in their interaction with other cells, we

studied with immunohistochemical methods the expression of MHC molecules on HNSCC and also on the line-10 guinea-pig tumor. If cytotoxic $CD8^+$ T lymphocytes are involved in the PEG-IL-2-induced antitumor effect, like suggested above, then high MHC class I expression by the tumor would be favorable. In primary HNSCCs of various sites a relatively high MHC class I expression was found, especially in tumors from the oral cavity (chapter 3). In view of the observed antitumor efficacy, a surprisingly low MHC class I expression was observed in the PEG-IL-2-treated line-10 guinea-pig tumor (chapter 6). In the 4 patients with the most favorable course after PEG-IL-2 therapy also a low MHC class I expression was found in their primary tumors or previous recurrences (chapter 7). Although the latter only provides indirect evidence, these findings do not support a major role for $CD8^+$ T cell-mediated tumor cell lysis. We did not observe changes in MHC class I or II expression by tumor cells induced by locoregional IL-2 (chapter 4) or PEG-IL-2 (chapters 6,7) administration.

The necessity to administer IL-2 and PEG-IL-2 directly at the tumor site to induce an effective host antitumor immune response, as demonstrated in this (chapter 5) and previous experimental studies, suggested that the effect was generated at the tumor site. This was the main reason for applying PEG-IL-2 intratumorally in patients in the same dose as found optimal in guinea-pigs. There are no arguments to suppose that the working mechanism of PEG-IL-2 is basically different from that of IL-2. The enhanced efficacy in the line-10 tumor was assumed to be related to differences in kinetics. To confirm this, radiolabeled IL-2 and PEG-IL-2 were injected intratumorally in guinea-pigs and the radioactivity at several sites was measured over time (chapter 8). No evidence was found for prolonged retention of PEG-IL-2 in the tumor or the regional lymph nodes. The blood levels for PEG-IL-2, however, were higher. This raises questions about the contribution of circulating, systemic IL-2 activity to the antitumor effect of locally injected (PEG-)IL-2.

Reviewing these and previous investigations (chapter 2) on anticancer immunotherapy with IL-2 it can be concluded that a host immune response towards the tumor can be elicited in experimental models by protracted peri- or intratumoral application of IL-2. In established disease PEG-IL-2 might be more effective than IL-2. This immune response can mediate local and distant tumor regression, and may encompass protective immune memory. Both T-cell mediated and non-specific mechanisms appear to be involved in this therapeutic effect. Production of interferon-γ has been shown to play a role. Immunohistochemically detectable MHC class I expression by the tumor cells appears not to be

necessary. In several animal studies a dose-response relation has been observed in the low to intermediate IL-2 dose range.
Whether a systemic immune response can be elicited by intra- or peritumoral IL-2 administration in cancer patients is not known. Occasionally, HNSCC patients with limited local or regional disease appear to benefit from locoregional IL-2 or PEG-IL-2 therapy. Local tumor regression has been observed. The therapy is well tolerated. So far, there is no evidence in HNSCC patients that high doses of locally administered IL-2 are more effective than low doses.

The mechanism of local IL-2 immunotherapy is still incompletely understood, as are the prerequisites for a favorable response. Most experimental studies have been performed in early-stage disease, while advanced disease is common in the clinical situation. Depressed host immune responsiveness, as demonstrated in advanced tumor-bearing hosts, might contribute to the presently observed restricted efficacy of local IL-2 therapy in cancer patients. Local IL-2 therapy might be more appropriate for patients with minimal disease, as an adjuvant to conventional therapy.

Possible targets for future research include:
1. Factors related to tumor-host interaction: tumor-induced immune suppression (depressed T cell responsiveness), tumor-specific antigens, antigen presentation, co-stimulatory signals (B7/CD28), tumor micro-environment (antigen-presenting cells).
2. Better understanding of the mechanisms triggered by local IL-2: involved immune-competent or inflammatory cells, secondary cytokines, systemic effects, IL-2 receptor expression by tumor cells.

Samenvatting

De behandeling van kanker steunt op drie pijlers, te weten chirurgie, radiotherapie (bestraling) en chemotherapie (toediening van tumorcel-dodende medicijnen). De laatste 10 jaren is immunotherapie in ontwikkeling als een potentiële vierde behandelingsmogelijkheid van kanker. Bij immunotherapie wordt beoogd het lichaamseigen afweersysteem (immuunsysteem) aan te zetten kankercellen als zodanig te herkennen en af te breken. In het immuunsysteem werken een groot aantal cellen (meest witte bloedcellen) samen, zoals: T lymfocyten, B lymfocyten, NK (natural killer) cellen, monocyten, macrofagen en granulocyten. Deze cellen kunnen elkaar signalen geven door middel van eiwitten die ze zelf maken, zogenaamde cytokinen.
Interleukine-2 (IL-2) is een dergelijk cytokine dat gemaakt wordt door T lymfocyten en dat van nature een belangrijke rol speelt bij het in gang zetten van een immunologische (afweer) reactie. Tevens blijkt IL-2 in staat om T lymfocyten en NK cellen aan te zetten tot tumorcel-dodende (cytotoxische) activiteit. Sinds IL-2 in het laboratorium geproduceerd kan worden door middel van de zogenaamde recombinant DNA techniek is het uitgebreid onderzocht op zijn waarde voor de behandeling van patiënten met kanker. Doorgaans wordt het in hoge doses met een infuus in de bloedbaan toegediend. Hiermee zijn opmerkelijke resultaten bereikt, alhoewel slechts bij een klein deel van de patiënten (met name met melanomen en niercelkanker) en ten koste van ernstige bijwerkingen. Het precieze werkingsmechanisme van deze behandeling is niet bekend.
Dit proefschrift handelt over immunotherapie met lokale IL-2 toediening, dat wil zeggen toediening direct op de plaats van de tumor. Het onderzoek is met name gericht op toepassing van IL-2 bij patiënten met plaveiselcelcarcinomen van het hoofd-halsgebied. Dit zijn tumoren die ontstaan uit de slijmvliezen van mond- of keelholte. Door de oppervlakkige lokalisatie, de voornamelijk lokale en regionale tumor-uitbreiding en de nabijheid van lymfatisch weefsel lijken deze tumoren relatief goed geschikt voor lokale immunotherapie.

In *hoofdstuk 2* wordt nader ingegaan op de redenen om IL-2 lokaal toe te dienen in plaats van systemisch (in de bloedbaan). Van nature werkt IL-2 over een hele kleine afstand, namelijk op de plaats waar het ook gemaakt wordt en komt het niet in grote hoeveelheden in het bloed. Als het in de bloedbaan wordt toegediend wordt het daaruit ook weer snel door de nieren verwijderd. Bij lokale toediening van IL-2 zou met een lagere dosis volstaan kunnen worden, hetgeen de bijwerkingen van de behandeling belangrijk zou kunnen verminderen. Daarnaast zijn in veel tumoren lymfocyten aanwezig waarvan in het laboratorium is aangetoond dat ze een potentieel hogere anti-tumor activiteit hebben dan

lymfocyten uit de bloedbaan. Deze tumor-infiltrerende lymfocyten (of TIL cellen) zouden door lokaal toegediend IL-2 rechtstreeks geactiveerd kunnen worden. Verder is door verschillende onderzoekers aangetoond dat bij dieren met tumoren in een vroeg stadium een specifieke, tegen de tumor gerichte, immunologische reactie opgewekt kan worden door aanwezigheid van IL-2 op de plaats van de tumor.

In *hoofdstuk 3* wordt een zijpad bewandeld. Bij lokale immunotherapie met IL-2 spelen T lymfocyten mogelijk een belangrijke rol. Een voorwaarde voor interactie tussen T lymfocyten en tumorcellen is dat de tumorcellen zogenaamde MHC moleculen (bij de mens: HLA antigenen) op hun oppervlak tot expressie brengen. Het is bekend dat tumorcellen wat betreft HLA expressie vaak afwijken van normale cellen. Door middel van een immunohistochemische kleurmethode en microscopisch onderzoek hebben wij de HLA expressie van hoofd-halscarcinomen onderzocht. Er werd een hoge HLA klasse I expressie gevonden, met name in tumoren uitgaande van de mondholte. Dit suggereert dat deze tumoren gevoelig zouden kunnen zijn voor cel-dodende (cytotoxische $CD8^+$) T lymfocyten. De hier beschreven techniek is later toegepast op tumorweefsel van patiënten behandeld met IL-2.

In *hoofdstuk 4* wordt een studie beschreven met perilymfatische injecties met een hele lage dosis IL-2 bij patiënten met hoofd-halscarcinomen. Perilymfatische IL-2 injectie wil zeggen toediening rondom de lymfeklieren die gelegen zijn in de buurt van de tumor en is gericht op activering van lymfocyten in die lymfeklieren. Het gebruikte behandelschema was gebaseerd op een eerder gepubliceerd kleinschalig onderzoek, waarbij positieve resultaten waren bereikt. Vijftien nog niet eerder behandelde patiënten met zeer grote en daardoor niet te genezen hoofd-halstumoren kregen, nadat zij daarvoor toestemming gegeven hadden, gedurende 10 dagen een perilymfatische IL-2 injectie. Uitwendig was er géén effect op de tumor en ook microscopisch onderzoek van weefselstukjes (biopten) uit de tumor toonde geen verschil ten opzichte van de situatie voorafgaande aan de behandeling. Er traden geen bijwerkingen van de behandeling op. Het verschil in resultaat ten opzichte van de eerder gepubliceerde studie heeft waarschijnlijk te maken met de grootte van de tumoren.

In *hoofdstuk 5* worden een aantal experimenten beschreven verricht bij cavia's met tumoren op de flank (line-10 tumoren). Het doel van deze studies was meer informatie te krijgen over de optimale dosis en toedieningsweg van IL-2 bij lokale immunotherapie. Behalve IL-2 werd ook PEG-IL-2 getest. Dit is IL-2 waaraan lange ketens van de stof polyethyleen

glycol zijn gekoppeld, die zorgen voor een betere oplosbaarheid en een tragere verwijdering van de stof uit de bloedbaan. Het bleek dat cavia's met uitwendig voelbare tumoren op de flank en microscopisch waarneembare uitzaaiingen in de regionale lymfeklieren genezen konden worden met herhaalde injecties met PEG-IL-2, rechtstreeks toegediend in de tumor (intratumoraal). Als deze dieren vervolgens op de andere flank opnieuw line-10 tumorcellen ingespoten kregen, dan werden die afgestoten. Dit geeft aan dat beschermende immuniteit tegen de tumor was ontwikkeld. PEG-IL-2 was sterker werkzaam dan IL-2 en hoefde minder vaak toegediend te worden. Intratumorale toediening was essentieel voor een goed effect.

In *hoofdstuk 6* wordt verslag gedaan van een microscopisch onderzoek van de caviatumoren in verschillende fasen van behandeling met intratumorale PEG-IL-2 injecties in vergelijking met niet-behandelde tumoren. Voor de start van de behandeling waren er lymfocyten in de directe omgeving van de tumor aanwezig. Bij een immunohistochemische kleuring bleek het met name te gaan om $CD4^+$ T lymfocyten. Mogelijk is de gunstige reactie opgewekt door de PEG-IL-2 injecties een gevolg van activering van deze lymfocyten. Tijdens de PEG-IL-2 behandeling ontwikkelde zich een uitgebreide ontstekingsreactie met T lymfocyten, eosinofiele granulocyten, macrofagen en fibroblasten rondom tumor en regionale lymfeklieren. Met name $CD8^+$ T lymfocyten drongen daarbij door tot in de tumor, hetgeen suggereert dat cytotoxische T lymfocyten een rol spelen bij het opruimen van de tumor. De door ons waargenomen lage MHC klasse I expressie op de line-10 tumorcellen is hiermee echter in tegenspraak, aangezien MHC klasse I expressie op de tumorcellen noodzakelijk is voor herkenning door $CD8^+$ T lymfocyten.

Het behandelschema waarmee optimale resultaten werden behaald bij de cavia's is vervolgens onderzocht in de patiëntenstudie die beschreven is in *hoofdstuk 7*. Hiervoor werden patiënten geselecteerd bij wie na een aanvankelijk succesvolle behandeling met operatie en/of bestraling de tumor in het hoofd-halsgebied opnieuw was teruggekomen en voor wie er geen uitzicht meer was op genezing. Negentien patiënten gaven hun toestemming voor deelname aan dit onderzoek en kregen 3 maal per week een injectie met een middelmatige dosis PEG-IL-2 rechtstreeks in de tumor, in kuren van 4 weken. De behandeling werd poliklinisch gegeven en werd goed verdragen. Er traden geen algemene ziekteverschijnselen op. Wel kregen 8 patiënten tijdelijk een zwelling met roodheid van het gelaat en/of de hals aan de kant van de injecties. Bij 7 van deze 8 patiënten werd tevens een tijdelijke stijging van het aantal eosinofiele granulocyten in het bloed waargenomen. Het resultaat van de behandeling bestond eruit dat bij 1 patiënt de tumor volledig

verdween. Deze patiënt bleef 91 weken vrij van tekenen van ziekte. Bij 3 patiënten werd gedurende enige tijd (23 tot meer dan 57 weken) stilstand in de tumorgroei waargenomen. Deze 4 patiënten hadden allen één enkele tumorlokalisatie in de hals die van een beperkte omvang was. Opvallend was dat bij alle 4 de patiënten tijdens behandeling de bovengenoemde lokale zwelling en roodheid optrad en dat zij de allerhoogste aantallen eosinofiele granulocyten in het bloed vertoonden.

Wij veronderstelden dat PEG-IL-2 na intratumorale toediening langer in de tumor en/of de regionale lymfeklieren aanwezig bleef dan IL-2. Om dit nader te onderzoeken werd een biodistributie studie uitgevoerd bij cavia's. Deze studie is beschreven in *hoofdstuk 8*. IL-2 en PEG-IL-2 werden hiervoor radioactief gemerkt en intratumoraal ingespoten in line-10 cavia-tumoren. Vervolgens werd op verschillende tijdstippen vastgesteld hoeveel activiteit aanwezig was in diverse weefsels. De uitkomsten leverden geen steun op voor de bovengenoemde veronderstelling. Wel bleek PEG-IL-2 na intratumorale toediening hogere concentraties in het bloed op te leveren dan IL-2. De vraag is in hoeverre dit bijdraagt aan de sterkere anti-tumor werking van PEG-IL-2 dan van IL-2 na intratumorale toediening.

In *hoofdstuk 9* worden nog eens kort de resultaten van de hier beschreven en door anderen gepubliceerde onderzoeken met betrekking tot immunotherapie met IL-2 bediscussieerd. Geconcludeerd wordt dat door IL-2 op de plaats van de tumor te brengen in experimentele dierentumoren een immunologische reactie gericht tegen de tumor opgewekt kan worden, die zowel lokaal als op afstand kan leiden tot afbraak van tumor. Zowel T lymfocyten als andere, niet-specifieke immuuncellen lijken betrokken bij dit effect. In reeds uitgegroeide tumoren lijkt PEG-IL-2 effectiever dan IL-2. Of een dergelijke effectieve systemische immunologische reactie ook bij kankerpatiënten opgewekt kan worden door intratumorale IL-2 toediening is nog de vraag. Patiënten met hoofd-halscarcinomen van beperkte omvang blijken in een enkel geval voordeel te hebben van lokale (PEG-)IL-2 behandeling. Deze behandeling wordt goed verdragen. Er zijn tot nu toe geen aanwijzingen verkregen in patiëntenstudies dat hogere doses lokaal toegediend IL-2 tot betere resultaten leiden. Het precieze werkingsmechanisme van lokale immunotherapie met IL-2 in nog niet bekend. Dierexperimenten hebben meestal betrekking op tumoren in een heel vroeg stadium, terwijl er bij patiënten altijd sprake is van voortgeschreden ziekte. Een onderdrukt immuunsysteem, zoals beschreven bij kwaadaardige ziekten in een gevorderd stadium, zou mede verantwoordelijk kunnen zijn voor de beperkte effectiviteit van lokale IL-2 therapie bij patiënten met kanker.

Appendix

Jakobsson score,

histopathological grading scheme for squamous-cell carcinoma[1]

Histopathologic parameter	Score	
Cytoplasmic keratinization	1	High degree, well-formed pearls
	2	Moderate, 20-50% of cells, attempts at pearl formation
	3	Poor, 5-20% of cells with suggestion of keratinization
	4	No evidence of keratinization
Nuclear differentiation	1	Few enlarged nuclei, >75% mature appearing
	2	50-75% mature appearing nuclei
	3	Considerable nuclear pleomorphism, 25% mature appearing
	4	Anaplastic tumor
Mitosis, average number/HPF	1	0-1
	2	2-3
	3	4-5
	4	>5
Inflammatory response	1	Continuous rim
	2	Patchy rim
	3	Occasional patch
	4	None
Vascular/lymphatic invasion	1	Not identified
	4	Identified
Pattern of invasion	1	Pushing borders
	2	Solid cords
	3	Thin irregular cords
	4	Single cells

HPF, high-power field, 40x.

[1] Crissman *et al.*, *Cancer* 1984; 54:2995-3001.

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Het dankwoord is één van de best gelezen delen van een proefschrift, zoals u als lezer wellicht kunt beamen. Een goede zaak, want het doet recht aan de inspanning van de vele medewerkers die een promotie-onderzoek doorgaans kent. Zo ook dit onderzoek, dat tot stand is gekomen door samenwerking van diverse afdelingen, deels binnen en deels buiten het Academisch Ziekenhuis Nijmegen (AZN). Graag wil ik allen danken die hieraan hebben bijgedragen:

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Curriculum vitae

Vera Mattijssen werd op 27 januari 1960 te Bemmel geboren. In 1978 behaalde zij het Gymnasium-β diploma aan het Katholiek Gelders Lyceum te Arnhem. Aansluitend studeerde zij Geneeskunde aan de Katholieke Universiteit te Nijmegen. Het artsexamen werd behaald in januari 1986. Vanaf 1 februari 1986 was zij werkzaam als arts-assistent in opleiding tot internist, aanvankelijk in het St. Maartens Gasthuis te Venlo (opleider: Dr. J.J. Mattousch) en vanaf 1 februari 1988 op de afdeling Inwendige Ziekten van het St. Radboudziekenhuis te Nijmegen (opleiders: Prof. Dr. A. Van 't Laar en Prof. Dr. J.W.M. Van der Meer). Van 1 juni 1989 tot 1 juni 1993 werd de opleiding onderbroken en werkte zij op de afdeling Medische Oncologie van het St. Radboudziekenhuis (hoofd: Prof. Dr. D.J.Th. Wagener). In die periode werden onder meer de onderzoeken verricht die beschreven zijn in dit proefschrift. In mei 1991 bezocht zij het Massachusetts General Hospital te Boston (USA) in het kader van een uitwisselingsprogramma voor arts-assistenten. De opleiding tot internist zal worden afgerond op 1 augustus 1994.

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[1] The Journal of Immunotherapy was formerly called Journal of Biological Response Modifiers.

Stellingen

behorende bij het proefschrift

Locoregional immunotherapy with interleukin-2 in head and neck squamous-cell carcinoma
Preclinical and clinical studies

Vera Mattijssen, 10 mei 1994

1. In diverse experimentele tumormodellen is de werkzaamheid aangetoond van intra- of peritumorale toediening van relatief lage doses IL-2 aangaande het opwekken van een effectieve antitumor immuunrespons.

2. Herhaalde intratumorale injectie van PEG-IL-2 kan cavia's met een palpabele line-10 tumor op de flank en micrometastasen in de regionale lymfklieren genezen.

3. Histologische analyse van line-10 tumorregressie onder invloed van intratumorale PEG-IL-2 toediening suggereert dat een complex van immuuncompetente cellen betrokken is bij dit antitumor effect.

4. Op basis van de mogelijkheid tot interactie met $CD8^+$ T lymfocyten lijkt expressie van MHC klasse I door tumorcellen van belang bij (locale) immunotherapie met IL-2. Met immunohistochemisch onderzoek is hiervoor in therapie-experimenten evenwel nog geen duidelijke bevestiging gevonden.

5. Als circulerend IL-2 bijdraagt aan het antitumor effect van intratumorale (PEG-)IL-2 therapie dan heeft dat consequenties voor de vertaling van dierexperimentele behandelschema's naar de humane situatie.

6. Het "garbage effect" in experimentele immunotherapie ("the observation that if a procedure can make animals sick enough, even their tumors will suffer") is niet van toepassing op locale IL-2 therapie.
   J. Vaage, Immunol Lett 1989; 21:275.

7. Een hoog percentage tumorcellen met membraneuze expressie van het epitheliale adhesiemolecuul E-cadherine is gerelateerd aan een gunstig klinisch beloop bij hoofd-hals plaveiselcelcarcinomen.

8. Herhaling van instructie in primaire reanimatie om de zes maanden houdt de vaardigheden op een adequaat niveau.
Bart Berden et al., BMJ 1993; 306:1576-7.

9. De voortschrijdende (sub)specialisatie stelt hogere eisen aan de communicatie tussen specialist en huisarts, zowel algemeen vakinhoudelijk als ook toegespitst op de individuele patiënt. In de opleiding tot specialist dient hieraan bijzondere aandacht te worden besteed.

10. Opleiding van vrouwen is het beste anti-conceptiemiddel.
Margaret Catley-Carlson, voorzitter Population Council.

11. Het in het huidige Nederlandse bestel gelijkstellen van economische groei aan groei van welvaart getuigt van onvoldoende begrip voor mens en milieu.

12. De wijze waarop in proefschriften partners doorgaans worden bedankt voor hun schijnbaar onvoorwaardelijke steun en begrip doet vrezen voor een verengd bewustzijn van promovendi ten tijde van de afronding van hun onderzoek.